

GENOTYPE EFFECTS IMPACT LIPIDS AND ORGAN WEIGHTS IN FEMALE MICE LACKING CAROTENOID CLEAVAGE ENZYMES

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Nutritional Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

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Abstract

Carotenoids are very abundant in nature and their consumption has numerous positive health outcomes. There are two known enzymes that are responsible for cleaving carotenoids into their metabolites. β -carotene is cleaved by carotenoid-15,15'-oxygenase (CMO-I), to eventually form biologically-active retinoids. Lycopene is a non-provitamin A carotenoid and the most abundant carotenoid in tomatoes. Lycopene is a poor substrate for CMO-I, but our lab along with other has proposed that the enzyme carotenoid 9'10'-monooxygenase (CMO-II) can oxidatively cleave lycopene. In order to assess the impact of dietary carotenoids and their metabolites on lipid metabolism in female mice, CMO-I KO, CMO-II KO or wild type (WT) mice, 29-31 weeks old, were fed the following AIN-93G based diets for either 4 or 30 days: lycopene beadlet, 10% tomato powder, and their respective controls. We hypothesized that mice lacking either carotenoid cleavage enzyme would have altered serum and hepatic lipids compared to WT mice while lycopene or tomato powder might modulate these effects. Our data demonstrate that a lack of CMO-I or CMO-II altered reproductive organ weights and lipid status, and feeding carotenoid-containing diets had modest impact on lipid metabolism in these mice.

This thesis is dedicated to my family. Thank you for your continuous support and encouragement. I would not have been able to make it through this process without any of you.

Acknowledgments

I would like to thank everyone who was a part of my graduate school experience. I would like to especially thank Dr. Erdman for letting me join your lab as an undergraduate student, and allowing me continue on as a graduate student. I am very grateful to have been a part of your lab, and for having such an experienced mentor.

I would like to thank the following people for their help with my research, my writing and their encouragement throughout my graduate school education. Nikki Ford, thank you for developing the basis for my research project, and for your guidance during the beginning of my graduate career. I would also like to thank Nancy Engelmann, Lauren Conlon, Peter Lu and Ann Liu for their guidance and expertise in HPLC extractions. Additionally, I would like to thank Krystle Zuniga and Josh Smith for your insightful critiques of my writing and your help with understanding complicated concepts within my research and nutrition in general. I thank you all for your contributions during this process.

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Chapter I

Literature Review

Introduction

Carotenoids occur throughout nature, and more than 600 carotenoids have been discovered. Most carotenoids are derived from the same C₄₀, tetraterpene structure and are commonly produced in the photosynthetic tissues of plants and algae. They can be grouped either as hydrocarbons called carotenes, or their oxygenated derivatives called xanthophylls (1). Carotenoids are not known to be produced by mammals, yet their consumption from the diet is important for vitamin A production, because some carotenoids have provitamin A activity (β -carotene, α -carotene, and β -cryptoxanthin). Provitamin A carotenoid consumption helps to alleviate vitamin A deficiency symptoms which can lead to blindness, increase mortality, impaired immune function, and numerous other negative health outcomes. Carotenoids may also have anticancer activity and have been shown to influence the risk and progression of cancers of the lung, breast, prostate, cervix and ovary (2). There is high variability in the provitamin A activity of β -carotene (3), which could possibly be due to differences in intestinal conversion of β -carotene to vitamin A, inefficient incorporation of β -carotene into chylomicrons or higher rates of β -carotene clearance as a result of abnormal lipoprotein metabolism.

Carotenoid Cleavage Enzymes

Carotene-15,15'-monooxygenase

In order for provitamin A carotenoids to be converted into vitamin A they must first be cleaved by carotene-15,15'-monooxygenase (CMO-I), an enzyme initially identified from the supernatant solution of rat liver and intestine (4). Cleavage occurs at the central carbon double

bond, and theoretically creates 2 molecules of retinal, which can be reduced to retinol (5). Additionally researchers have found that oxygen is necessary in order for this cleavage to take place, and that CMO-I is a nonheme iron-containing enzyme (4, 5). Cleavage of β -carotene is an important source of retinoic acid in various tissues suggesting that this cleavage plays a vital role in retinoid homeostasis (6). CMO-I has been identified as a cytoplasmic enzyme (7). CMO-I is expressed in the human intestinal tract with the highest mRNA expression being in the jejunum. CMO-I is also expressed in many other tissues including the liver, kidney, prostate, testis, ovary, colon and skeletal muscle (7).

Carotene-9'10'-monooxygenase

Another carotenoid cleavage enzyme, carotene-9'10'-monooxygenase (CMO-II), is responsible for the asymmetric cleavage of β -carotene to form β -apo-10'carotenal and β -ionone (8). CMO-II cleaves at a different pH than CMO-I (8.5 vs. 7.7) which suggests that these enzymes perform different carotenoid cleavage functions (9). CMO-II was identified as a mitochondrial protein and is thought to protect against oxidative damage caused by excess carotenoid accumulation (10). mRNA expression of CMO-II was found in the same tissues as CMO-I (small intestine, liver, kidney, and testes), and also in spleen, brain, lung and heart (7, 8)

Single nucleotide polymorphisms

Two nonsynonymous single nucleotide polymorphisms (SNPs) were identified (R267S and A379V) in the human CMO-I coding region, and the variant allele frequencies in a female Northern European cohort of these SNPs were 42 and 24% respectively (11). Leung et al. analyzed plasma samples from 28 female volunteers and found that those with the A379V variant allele, and the combined R267S and A379V variant alleles had significantly higher concentrations of fasting plasma β -carotene. These findings are important because in double

tracer studies, 27-45% of subjects were identified as poor converters of β -carotene and these and other SNPs are most likely the reasons for this poor converter phenotype (12-14).

Recently, SNPs within the human CMO-II gene have been described (15). Female variant allele carriers of common CMO-II SNPs have been found to have reduced fasting HDL-cholesterol concentrations compared to wild-type carriers (abstract data) (16).

Lycopene

Absorption and Transport

Lycopene, a non-provitamin A carotenoid, is the most abundant carotenoid in tomatoes and is responsible for their red pigment (17). In order for lycopene, along with other dietary carotenoids, to be absorbed optimally a few key events must occur; 1) the food matrix must be digested for release of the carotenoids, 2) lipid micelles must be formed, 3) carotenoids must be taken up into the intestinal mucosal cells, and 4) carotenoids and their metabolic products must be transported to the lymphatic circulation (18). In order to digest the food matrix of lycopene-containing foods, mild heating has been found to increase the plasma appearance of lycopene from tomato juice compared to tomato juice that has not been heated prior to ingestion (19). In addition, incorporation of fat into a carotenoid-containing meal can aid in absorption by stimulating bile production, and increasing micelle formation. It has also been suggested that *cis*-isomers of lycopene are preferentially absorbed when compared to all-*trans* lycopene isomers (19). Another study that supports this suggestion, found that *cis*-isomers preferentially accumulated in prostate tissues of men (20). Once lycopene is absorbed into the mucosal cells it is then transported in the lymph system to the liver for repackaging into lipoproteins like very low density lipoproteins (VLDL) and low density lipoproteins (LDL). Fasting blood analysis revealed that lycopene is mainly carried in the LDL fraction (21).

Cleavage

In order for cleavage of lycopene by CMO-I to occur, there must be very high concentrations of lycopene present (6). Incubation of lycopene and a *Drosophila* homologue of CMO-I in rat liver and intestine, showed no lycopene cleavage products (22). In addition to a lack of activity of human CMO-I on lycopene, researchers have also seen no activity of human CMO-I on zeaxanthan (7). As a result of this data, researchers concluded that another enzyme must then be responsible for cleavage of lycopene. Consequently, CMO-II has been shown to cleave lycopene in *E.coli* strains transfected with either mouse or ferret CMO-II (8, 9). Animal studies from our laboratory support this theory that CMO-II cleaves lycopene, as CMO-II KO mice fed lycopene or tomato powder had greater tissue lycopene accumulation compared to wild-type mice (23, 24). In addition, *in vitro* evidence suggests that CMO-II cleaves xanthophylls at the 9',10' carbon double bond (10).

Action

Lycopene has been classified as an antioxidant and has been shown to scavenge the ABTS^{•+} radical cation more extensively than β -carotene in an *in vitro* antioxidant activity study (25). Lycopene's action as an antioxidant has been associated with a lower incidence of metabolic syndrome (26). Metabolic syndrome was initially described as a group of diseases including glucose intolerance, dyslipidemia, high blood pressure, and coronary heart disease (27) and now includes excess weight, low concentrations of HDL and hypertriglyceridemia (28). Sluijs et al. found that with higher intake levels of lycopene, there was a decreased trend in prevalence of metabolic syndrome (26). However, in another study that analyzed the Third National Health and Nutrition Examination Survey (NHANES III) data on the association of

metabolic syndrome and antioxidant concentrations, lycopene concentrations were not associated with a decrease in metabolic syndrome (28).

As mentioned before, carotenoids have been shown to have anticancer abilities (2). In epidemiological studies high levels of serum lycopene were associated with a reduced risk of prostate cancer (29, 30). Our lab has demonstrated a possible mechanism in which lycopene, and its metabolites, decrease prostate cancer progression. Androgen-independent prostate cancer cells (DU145 cells) were treated with a supraphysiological doses of either lycopene or apo-12'-lycopenal. A reduction in the proliferation of these cells was seen 72 hours post treatment of either lycopene or apo-12'-lycopenal. The mechanism, to some extent, was identified as inhibition of normal cell cycle progression (31). Many other studies have investigated additional mechanisms of lycopene's action in reducing prostate cancer. These mechanisms include the ability to increase the cell-cell communication pathway by an increasing the gap junction communication protein, connexin 43 (32, 33). Our lab, however, failed to find that lycopene affected connexin 43 (31).

PPARs and their target genes

Peroxisomal proliferator-activated receptor (PPAR) gamma and alpha are part of the same nuclear hormone receptor superfamily and are ligand dependent transcription factors. PPAR γ is necessary for fat cell formation (34) and, as a result, PPAR γ is the target of glitazones, especially thiazolidinediones (TZDs), which are used as antidiabetic agents (35). PPAR α is responsible for modulating oxidation of fatty acids in the liver (36). Fibrates, which are PPAR α activators, are used to protect against coronary artery disease by increasing gene expression of apolipoproteins in HDL, resulting in an increase in reverse cholesterol transport (reviewed in (36)).

A nuclear factor, adipocyte regulatory factor 6 (ARF6), was found to bind to a peroxisome proliferator response element (PPRE)-like sequence in the fatty acid binding protein 4 (FABP4) promoter region, and was shown to enhance transcription of FABP4 (37-39). This factor was later discovered to be the PPAR γ and RXR α heterodimeric complex (40). Additionally, heterodimerization of PPAR γ and RXR α in the PPRE of the promoter region of CMO-I regulates the expression of this gene (41). These discoveries demonstrate the similarities between activation of FABP4 and CMO-I by PPAR γ and RXR α .

PPAR α was also found to heterodimerize with RXR α in the PPRE of the promoter regions of genes involved in β -oxidation, most notably acyl-CoA oxidase (ACOX) (42). PPAR α was observed to stimulate the promoter of ACOX by 3-fold when a PPAR α expression vector was co-transfected into HeLa cells with a rat ACOX gene promoter region (43).

Lycopene has been hypothesized to exhibit effects on PPAR γ expression. In one study, LNCaP (an androgen-dependent prostate cancer cell line) cells were treated with lycopene. After 24 hours of lycopene treatment, there was an increase in both protein and mRNA expression of PPAR γ (44). In another *in vitro* study, lycopene was also shown to reduce the intracellular levels of cholesterol in human macrophages by increasing PPAR γ mRNA expression, which enhanced the expression of ATP-binding cassette protein A1 (ABCA1) and caveolin-1 (cav-1), and as a result, decreased 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (45). Our lab also looked at lycopene's effects on PPAR γ mRNA expression, but found that lycopene feeding decreased PPAR γ mRNA expression in rat kidneys and adrenals (46).(42)

CMO-I KO and CMO-II KO mice phenotype

In addition to carotenoid metabolism, evidence suggests that the CMO-I enzyme is more broadly involved in lipid metabolism, and some of the actions of CMO-I may be independent of

carotenoid consumption. As detailed below, mice lacking the CMO-I enzyme have altered lipid metabolism. There are only a few papers published from studies on CMO-II KO mice and no details on altered lipid metabolism in this phenotype have been reported. CMO-I KO mice were developed by researchers at DSM Nutritional Products (Kaiseraugst, Switzerland)(47). CMO-II KO mice were developed by DSM and by the Institut für Immunbiologie at the University of Freiburg (Freiburg, Germany)(10).

Body and organ weights

In the literature there is evidence that the CMO-I genotype impacts body and tissue weights. Both male and female CMO-I KO mice were heavier upon completion of a cholesterol-supplemented lycopene-containing diet study compared to WT mice (24). In another study, female CMO-I KO mice, compared to WT mice, at the start of a study had lower average body weights, but these mice showed a tendency to gain more weight during a 14 week feeding period than WT mice (48). As a result, at the end of this study the CMO-I KO mice weighed the same as the WT mice. In an 8 week high fat feeding study, female CMO-I KO mice gained significantly more weight than female WT mice (47).

Reproductive organ weights have also been measured in CMO-I KO and CMO-II KO mice and have been found to be significantly different in weight as a percent of body weight. In one study from our lab, CMO-I KO female mice had significantly smaller uteri compared to WT mice independent of dietary carotenoids (24). In another study, which simultaneously compared the effects of two tomato carotenoid diets and two control diets, male CMO-II KO mice had smaller testes and seminal vesicles as a percentage of body weight compared to WT mice independent of tomato carotenoid feeding (23).

Hepatic Lipids

Hepatic lipids have also been measured in a few CMO-I KO studies and one study with CMO-II KO mice. Researchers were interested in determining if any phenotypic alterations, in addition to impaired β -carotene metabolism, were associated with a CMO-I deficiency (47). They found that when compared to WT mice, CMO-I KO mice accumulated more hepatic lipids when fed diets with β -carotene with a very low level of vitamin A (150 IU vitamin A/kg diet) (47). In order to determine if the development of fatty liver could be prevented in these CMO-I KO mice, Hessel and coworkers fed preformed vitamin A to 25-week old CMO-I KO mice and WT mice (14,000 IU vitamin A /kg diet). The CMO-I KO mice still had significantly elevated levels of lipids in the liver compared to WT mice and had significantly more triglycerides in their livers (47). These results were not reproduced in a study where cholesterol-containing diets were fed (1500 IU vitamin A/kg diet) (24).

In order to determine the hepatic lipid content in CMO-II KO mice, heterozygous (+/-) and homozygous (-/-) CMO-II KO mice and WT mice were fed a vitamin A deficient diet supplemented with a xanthophyll, zeaxanthin (0.05mg zeaxanthin/g diet). Zeaxanthin feeding induced hepatic lipid droplet formation in the livers of male and female CMO-II^{-/-} and CMO-II^{+/-} mice, but not in zeaxanthin/chow-fed WT mice nor in chow-fed CMO-II^{-/-} mice (10). These results suggest that supplementation with certain carotenoids may contribute to lipid accumulation in the liver in this phenotype.

Serum Cholesterol

There may also be a relationship between CMO-I genotype, dietary carotenoids, and serum cholesterol levels. Lycopene feeding significantly lowered serum cholesterol levels in CMO-I KO mice fed a cholesterol-containing diet compared to CMO-I KO mice fed a lycopene-

free, cholesterol diet (24). In another study both male and female CMO-I KO mice, under standard dietary conditions, did not show differences in serum cholesterol ester and triglyceride levels compared to WT mice (47). However, levels of unesterified serum free fatty acids (FFAs) were significantly elevated in these mice compared to WT mice. Interestingly, when Hessel and coworkers fed 20-week-old female CMO-I KO and WT mice a high fat (30% w/w) diet, serum cholesterol ester levels were significantly higher in CMO-I KO mice compared to WT mice. Serum FFAs were also increased in these mice. These researchers hypothesized that since the rate of hepatic uptake of FFA is directly proportional to serum concentrations of FFA, the increase in FFAs in the serum may explain why their CMO-I animals developed fatty liver (47).

PPARs and their target genes

As stated before, PPAR γ and RXR α heterodimerize in the PPRE of the promoter region of CMO-I and regulates its expression, and as a consequence there has been interest in the expression of PPAR γ and its target genes in CMO-I KO mice. One study fed either a β -carotene supplemented diet (150 mg β -carotene/kg diet) or a control diet to female CMO-I KO and WT mice for 14 weeks, and found β -carotene supplementation down-regulated mRNA expression of PPAR γ in WT mice, but not CMO-I KO mice. These researchers also determined that there was a decreased mRNA expression of lipoprotein lipase (LPL), a target of PPAR γ (48). Hessel and colleges also measured PPAR γ target genes in the adipose of their mice (47). They found mRNA expression of FABP4 to be significantly elevated in CMO-I KO mice compared to WT mice.

PPAR α is also a PPAR of interest because of its involvement in fatty acid catabolism. When the mRNA expression of PPAR α was measured in CMO-I KO and WT mice, researchers did not find any differences between genotypes (47). However, when the mRNA expression of

ACOX1, a PPAR α target gene, was measured in these same mice they found CMO-I KO mice to have significantly elevated levels compared to WT mice (47).

Aims of thesis

In summary, while CMO-I and II enzymes are known to play a primary role in cleavage of carotenoids, some evidence from mice lacking one of these enzymes suggests that these genes have pleiotropic effects ranging from lipid metabolism to reproductive organ growth. The goals of this thesis are to: (1) examine the role of CMO-I and II in whole body and reproductive organ growth and lipid metabolism in female mice and to (2) investigate differences in expression of PPAR γ and PPAR α and selected genes they regulate (3) determine if there is a modulating effect of tomato carotenoids, on serum and hepatic lipids. We hypothesized that CMO-I KO mice would have altered lipid metabolism compared to WT mice independent of dietary carotenoids based on the work by Hessel and coworkers (47). We also hypothesized that CMO-II KO mice would have altered lipid metabolism compared to WT mice and that tomato carotenoids would impact hepatic lipids based on the results from Amengual and colleagues (10). Since there were no prior papers published on CMO-II KO mice when this project began, we based this hypothesis on what was observed in CMO-I KO mice. Our goal for this work was to focus on female mice because this laboratory has yet to investigate the impact of lycopene or tomato powder on females, especially regarding reproductive organs weights and lipid metabolism.

Chapter II

Lycopene and Tomato Powder Marginally Impact Lipid Status in Female CMO-I KO and CMO-II KO mice

Abstract

The β -carotene central cleavage enzyme, carotenoid-15,15'-oxygenase (CMO-I) is responsible for cleaving the carotenoid β -carotene to form vitamin A and with further metabolism, the production of biologically-active retinoids. It was previously found that adult mice lacking this enzyme have altered lipid metabolism resulting in fatty liver development. Lycopene, a non-provitamin A carotenoid and the most abundant carotenoid in tomatoes, is a poor substrate for CMO-I. Instead, lycopene can be oxidatively cleaved by carotenoid 9'10'-monooxygenase (CMO-II). Lycopene has been shown in our lab to decrease the expression of peroxisome proliferator-activated receptor gamma (PPAR γ), which is essential for adipogenesis, in some rat tissues. Interestingly, there is a peroxisome proliferator response element (PPRE) in the promoter region of CMO-I. In order to assess the impact of dietary carotenoids and their metabolites on lipid metabolism, female CMO-I KO, CMO-II KO or wild type (WT) mice, 29-31 weeks old, were fed the following AIN-93G based diets for 4 or 30 days: lycopene beadlet, 10% tomato powder, and their respective controls. We hypothesized that mice lacking either carotenoid cleavage enzyme would have altered serum and hepatic lipids compared to WT mice while lycopene or tomato powder might modulate these effects. The results show greater hepatic lipid accumulation in both CMO-I KO mice and CMO-II KO mice compared to WT mice. There were some genotype and diet effects in serum and hepatic cholesterol in each mouse strain compared to WT mice. Furthermore, we found genotypic differences in mRNA expression of PPAR α in the liver and PPAR γ and FABP4 expression in the adipose tissue and the liver.

Reproductive organ weights, as a percent of body weight were smaller in CMO-I KO and CMO-II KO mice compared to WT mice. Interestingly, we found the reproductive capacity of our breeding colonies of CMO-II KO mice to be decreased (average~2 pups/ litter) compared to CMO-I KO mice (average~5 pups/ litter) fed a standard chow diet. These data demonstrate that a lack of CMO-I or CMO-II altered reproductive organ weights and lipid status, and feeding carotenoid-containing diets had modest impact on lipid metabolism in these mice.

Introduction

Hundreds of carotenoids have been discovered and are commonly produced in the photosynthetic tissues of plants. They can be grouped as either the hydrocarbon carotenes or xanthophylls (which are oxygenated carotenoids) (1). Provitamin A carotenoid (β -carotene, α -carotene, and β -cryptoxanthin) consumption helps to alleviate vitamin A deficiency symptoms which can lead to numerous negative health outcomes. Carotenoids, even non-provitamin A carotenoids like lycopene, may also have anticancer activity and have been shown to influence the risk and progression of cancers, most notably prostate cancer (2, 29, 30).

Mice lacking CMO-I and CMO-II, enzymes responsible for cleaving carotenoids centrally (4) and eccentrically, respectively (8), have been used as models to investigate biological effects of carotenoids and their metabolites. Carotenoid accumulation studies in mice lacking either CMO-I or CMO-II demonstrate differences in accumulation of lycopene depending on which carotenoid is ablated (23, 24). In addition to the results published on bioaccumulation of lycopene in these KO mice, investigators have noted the development of hepatic steatosis in both CMO-I KO mice (47) and CMO-II KO mice (10). Our lab is interested in the role of tomato carotenoids, especially lycopene, and their effects on various health outcomes such as prostate cancer (23, 49-51). Based on the literature, CMO-I KO mice and

CMO-II KO mice apparently have altered lipid metabolism and we were interested to investigate if feeding lycopene or tomato powder would modulate different measures of lipid status such as hepatic lipid deposition and lipid metabolism-related genes in our female CMO-I KO and CMO-II KO mice.

Materials and Methods

Animals

All animal handling and surgical procedures were approved by the University of Illinois Institutional Animal Care and Use Committee. The generation of CMO-I KO mice and CMO-II KO mice has been previously published (10, 47). Female C57BL/6 x 129/SvJ (F1) mice (n~80) were purchased at 4-8 weeks of age for use as wild-type (WT) mice from Jackson Laboratory (Bar Harbor, ME) and were kept in the University of Illinois animal facility and fed rodent chow until they reached the appropriate age to go on study (29-32 week of age). CMO-I KO (B6;129S6-Bcmo1tm1Dnp) mice and CMO-II KO (B6;129S6-Bcdo2tm1Dnp) mice were bred at the animal facility. Confirmation of the genotype was previously described by using genomic DNA from mouse tail biopsies and the Extract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO) (23). CMO-I KO and CMO-II KO mice were bred with siblings to ensure homozygous knock-outs. They were given food and water *ab libitum* before and during the studies. 25-30 week old female WT, CMO-I KO and CMO-II KO mice consumed an AIN-93G diet for one week prior to randomization onto experimental diets.

Diets

A powdered AIN-93G diet was used for the foundation of all diets. Fresh diet was provided every 48 hours and new diets were made monthly. The diets were stored in the dark at 4°C. For the lycopene diet, 1g of 10% water soluble lycopene beadlets/kg diet was added (DSM,

Basel, Switzerland). The lycopene diet for the 30 day study contained 152 ± 7 nMol lycopene/g diet and for the 4 day study there was 176 ± 34 nMol lycopene/g diet (**Table 1**). For the tomato powder diet, 10% tomato powder (Futureceuticals, Momence, IL) was added to the AIN-93G diet. The 30 day study tomato powder diet contained 181 ± 42 nMol lycopene/g diet and there was 235 ± 19 nMol lycopene/g diet in the 4 day study. Additionally, vitamin A levels were reduced to low, but not deficient levels of 1500 IU vitamin A (retinol) provided by retinyl palmitate per kg diet (NRC 2,400 IU/kg diet) in all diets to help optimize absorption of carotenoids as discussed previously (24, 47).

Study Design

When WT mice were received from Jackson Labs they were separated by sex and placed two to a cage. All mice were given rodent chow until they were placed on the AIN-93G acclimation diet one week prior to randomization to the feeding studies. We fed animals for both 30 and 4 days. We wanted to determine if feeding carotenoids for different lengths of time could show the same results. Additionally, we wanted to determine if any dietary results could be seen after just 4 days of feeding. For both the 30 day and 4 day studies, mice were randomly assigned to one of four experimental tomato carotenoid-containing diets; 10% tomato powder, or 10% water-soluble lycopene beadlet or their respective controls (**Figure 1**). For statistical purposes the tomato powder group was compared to its AIN-93G control group while the lycopene beadlet-fed group was compared to their placebo beadlet control group (n~10 per genotype/diet/study). Mice were weighed every two days while on the 30 day study and mice were weighed daily for the 4 day study. At the conclusion of the studies, mice were fasted 3 hours prior to sacrifice. The mice were then asphyxiated by CO₂ and cardiac puncture was used to remove blood. Blood was placed on ice and protected from the light. Serum was separated

from the collected blood via centrifugation (1500xG for 20min at 4°C). Liver, spleen, kidneys, uteri, ovaries, gonadal and perirenal adipose tissue (visceral adipose) were removed, frozen in liquid nitrogen and then stored at -80°C. A small portion of the liver was saved in 10 % formalin (Sigma-Aldrich, St. Louis, MO) and after 24 hours at room temperature, the samples were placed in the refrigerator in 70% ethanol before transfer to the Comparative Biosciences Histology Laboratory at University of Illinois-Urbana/Champaign for histological analysis.

Hepatic Lipid Extraction

Total liver lipids were extracted using a modification of the Folch method (1, 24). Briefly, the liver sample (~0.3 g) was homogenized in chloroform: methanol (1:1) (Fisher Scientific, Pittsburg, PA.), and filtered by gravity using Whatman 110mm filter paper (Fisher Scientific). The mixture was washed with 0.29% sodium chloride solution, and then centrifuged at 183 x g for 5 min at 25°C. Chloroform was added to the mixture to ensure removal of lipids from the top layer. The sample was centrifuged again at 183 x g for 5 min at 25°C and the top layer was discarded. These steps were repeated 2 more times to guarantee complete isolation of lipids from our samples. The remaining solution was evaporated under a hood, placed to dry in a desiccator for at least 48 hours, and then weighed to determine total lipids.

Serum and Hepatic Cholesterol Analysis

Serum and hepatic total cholesterol were measured using an enzymatic colorimetric assay (Wako Chemicals USA, Richmond, VA). For hepatic cholesterol analysis, 1mL 10% Triton 100x (Sigma-Aldrich, St. Louis, MO) in isopropanol (Fisher Scientific) was added to our previously-extracted liver lipid samples and vortexed. Serum and liver lipid samples were then analyzed using the kit's instructions. Briefly, 20 µL of either serum or liver lipid extracts were mixed with 2 mL buffer (50µM Good's Buffer) mixed with the color reagent (cholesterol esterase,

cholesterol oxidase, peroxidase, DAOS, aminoantipyrine and ascorbate oxidase). The sample results were compared with a standard curve at concentrations of 100, 200, 397.4, 592.2 mg/dL. Plates were read at a wavelength of 600nm.

mRNA Expression Analysis

RNA isolation was done using a previously published method from manufacturer's instructions. Briefly, total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) in combination with homogenization. To determine the quality and concentration of the extracted mRNA, electrophoresis and spectrophotometry were used. If the mRNA was considered of good quality, cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). mRNA expression of these genes were measured via real-time PCR using Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Our primers were designed by Primer Express software and then validated. The primers that used β -actin (NM_007393.3) [Forward-5'-CGTGAAAAGATGACCCAGATCA-3' and Reverse-5'-AGCCTGGATGGCTACGTACATG-3'] as a control were PPAR γ (NM_011146.3) [Forward-5'-GCCACCAACTTCGGAATC-3' and Reverse-5'-TGCGAGTGGTCTTCCATCAC-3'] and ACOX1 (NM_027107.1) [Forward-5'-TCTGGAGATCACGGGCACTT-3' and Reverse-5'-TTCCAAGCCTCGAAGATGAGTT-3']. For analysis of PPAR α (NM_011144.6) [Forward-5'-CGA TGC TGT CCT CCT TGA TGA-3' and Reverse-5'-TC GCG TGT GAT AAA GCC ATT-3'] and FABP4 (NM_024406.2) [Forward-5'-AAACTGGGCGTGGAATTCG-3' and Reverse-5'-GGTTATGATGCTCTTCACCTTCCT-3'], L7A (NM_013721.3) [Forward-5'-CCTCTACAAGCGGCTCAAAGTC-3' and Reverse-5'-CCAGGGCCTGGGTGAAC-3'] was used as the control gene. Reactions were monitored by an ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA).

Tissue Carotenoid Extraction

All carotenoid samples were kept under yellow lights and on ice during the extraction procedure. Tissue samples were extracted using the procedure previously described with slight modifications (52). Briefly, 5 mL of ethanol containing 0.01% butylated hydroxytoluene (BHT) was added to 0.06-0.11g minced liver tissue or 0.2-0.7g adipose tissue. 1 mL 1:1 saturated (1g KOH to 1mL water) KOH was added and the samples were vortexed. A polystat model 12050-00 circulator rocking water bath (Cole Palmer, Court Vernon Hills, IL) at 60°C was used to saponify the samples for 30 minutes with intervals of vortexing occurring every 10 min. 2 ml of distilled water was added and carotenoids were extracted three times with 6 ml of hexane, dried under reduced pressure (SpeedVac concentrator, model AES1010, Savant, Milford, MA), and then topped with argon gas to prevent carotenoid degradation. For analysis, samples were reconstituted in methyl-*tert*-butyl ether and put into autosampler vials for use on a C30 HPLC column (18°C, 4.6 x 150 mm, 3µm; YMC, Wilmington, NC). A reverse-phase HPLC-PDA method was used to analyze the samples (53, 54). *All-trans* and 5-*cis* lycopene peaks were confirmed and quantified separately where possible and the rest of the *cis* lycopene peaks were quantified but their peak area counts were added together and labeled as “other *cis* lycopene”. Standards for individual carotenoids were used for comparison of elution times and absorption spectra.

Hepatic H&E Staining

For paraffin sections, livers were fixed in 10% formalin, transferred to ethanol after 24 hours and processed into paraffin blocks within a week of sacrifice. The embedded tissues were cut into 4-µm slices, mounted on slides, and dried in a 60°C oven. Slides were then stained using a standard hematoxylin and eosin (H&E) staining procedure used by the Veterinary Medicine

Pathology Lab at the University of Illinois-Urbana/Champaign. Representative histologic sections of each specimen (8/diet/genotype from the 4 day feeding study) were stained with H&E.

Statistical Analysis

Lycopene and tomato powder fed animals were statistically analyzed for their carotenoid content of the animal tissues. One way ANOVA was used for comparison of each KO genotype (CMO-I KO and CMO-II KO) with WT mice by SAS 9.1 (Cary, NC) with $\alpha = 0.05$. Tomato powder-fed KO animals were only compared with WT tomato powder-fed animals and lycopene beadlet-fed KO animals were only compared with lycopene beadlet-fed WT animals. Results were expressed as means \pm SEM. Statistical significance was considered when $P \leq 0.05$.

Organ weights and body weights were initially analyzed with a 2 x 2 factorial arrangement where each knock-out genotype (CMO-I KO, CMO-II KO) was compared to WT mice individually. There were no dietary effects seen with this statistical design so carotenoid-fed (tomato powder and lycopene beadlet) animal body weights and tissue weights, as a percent of body weight, were pooled with their respective control diet (AIN-93G or placebo beadlet diet) body weights and tissue weights, as a percent of body weight, for each genotype (except for livers in the 30 day study). Following pooling of groups, one way ANOVA was used for comparison of each KO genotype (CMO-I KO and CMO-II KO) with WT mice with $\alpha = 0.05$.

A 2 x 2 factorial design was used to analyze the hepatic and serum cholesterol, and gene expression data. Each knock-out genotype (CMO-I KO, CMO-II KO) was compared to WT mice individually. Carotenoid diets were only compared with their respective control diets (lycopene beadlet with placebo beadlet or tomato powder with AIN-93 G). Interactions between genotype and diet were investigated. All parameters were analyzed by analysis of variance (2-way

ANOVA). When it was possible, we blocked for cohort. When assumptions for ANOVA were violated, data were transformed by natural log or repeated measures were used. Results were expressed as means \pm SEM. Statistical significance was considered when $P \leq 0.05$.

Results

CMO-I KO mice and CMO-II KO mice differentially accumulate carotenoids

There were substantial genotype effects on lycopene accumulation in liver and adipose tissue following 30 days of tomato powder or lycopene beadlet feeding. CMO-I KO mice had significantly less total lycopene in their livers compared to WT mice when fed either 10% tomato powder or lycopene beadlet diets for 30 days (**Figures 2&3, Tables 2&3**). Tomato powder-fed CMO-I KO mice also accumulated less phytoene (PE) in their livers compared to WT mice (**Figure 2, Table 2**). In contrast, CMO-II KO mice had significantly more hepatic all-*trans*/5-*cis* and other *cis* isomer lycopene with tomato powder feeding and increased total lycopene accumulation when fed lycopene beadlet diet compared to WT mice. In the adipose tissue of the 30 day study animals, there were similar trends in lycopene accumulation as reported for the liver. CMO-II KO mice fed either lycopene beadlets or tomato powder had higher adipose total lycopene and all-*trans*/5-*cis* lycopene concentrations were significantly higher compared to WT mice (**Figures 4&5, Tables 4&5**). Other *cis* isomer lycopene accumulation was not statistically different between genotypes in the adipose tissue. In the serum of lycopene beadlet-fed animals, total lycopene accumulated significantly more in the CMO-I KO mice compared to WT mice (**Figure 6, Table 6**). For CMO-II KO mice, no significant differences in lycopene accumulation were noted when comparing WT mice with either carotenoid diet groups.

In the 4 day feeding study, lycopene-fed animals showed a similar genotypic carotenoid profile as the 30 day feeding study animals. CMO-I KO mice accumulated significantly lower levels of all-*trans*/5-*cis* lycopene in their livers compared to WT mice (**Figure 7, Table 7**). Total lycopene showed a trend of being higher in CMO-II KO mice and lower in CMO-I KO mice compared to their respective WT groups.

In addition to measuring lycopene, we also measured phytofluene (PF), PE and β -carotene (BC) in mouse tissues. BC was present in our tomato powder diet and in our liver tissues but it was at levels that were not quantifiable (data not shown). We were able to quantify PF and PE in the livers of our 30 day and 4 day studies but we could not identify PE and PF in the serum or the adipose tissue in all animals fed tomato powder diets. When looking at the PE peaks in our serum and adipose tissue we noticed a peak that had a similar retention time to PE and a maximum absorbance was similar to PE. However, the spectra did not show a carotenoid spectral fine structure expected of PE (**Figure 8**). These peaks were considered pseudo-PE and were not included in analysis.

CMO-II KO mice have reduced body weights

All mice were provided food and water *ab libitum* but feed intake was not measured. Prior to the 30 day experimental diet-feeding, the CMO-II KO mice were significantly smaller ($P < 0.001$) than WT mice and gained significantly less weight during the study (2.3 ± 0.4 g vs 4.8 ± 0.4 g) ($P < 0.001$). At the completion of the study there were no differences in body weight or organ weight (with the exception of liver) as a percent of body weight between diets in each genotype, so each carotenoid-containing diet was pooled with its control diet (tomato powder and AIN-93G, lycopene beadlet and placebo beadlet; results portrayed as “pooled” in Table 8 and 9). CMO-II KO mice were significantly smaller than WT mice (**Table 8**). CMO-II KO mice

were also smaller at the beginning ($P < 0.001$) and upon completion of the 4 day study compared to WT mice (**Table 9**). CMO-I KO mice were not significantly different in body weight or change in body weight compared to WT mice in either study.

We analyzed the visceral adipose weights (gonadal and perirenal adipose pads) of mice in our 30 day and 4 day studies. Visceral adipose weights, as a percent of body weight, were smaller in the CMO-II KO mice from the 30 day study and larger in the CMO-I KO from the 4 day feeding study mice compared to WT mice (**Tables 8&9**). In the 30 day study, the CMO-II KO mice had significantly larger livers, spleens and kidneys, as a percent body weight, compared to WT mice (**Table 8**). The livers, spleens and kidneys of the CMO-I KO mice were not significantly different from WT mice in weights, as a percent body weight, in the 30 day study. Conversely, in the 4 day study CMO-I KO mice had significantly smaller kidneys, as a percent body weight compared to WT mice (**Table 9**). CMO-II KO mice had significantly larger spleens, as percent body weight, when fed either the tomato powder or AIN-93 G diets compared to WT mice. Livers in the 4 day study were not different in sizes between genotypes.

In addition to a genotype effect in CMO-II KO mouse 30 day study livers there was a diet effect. Lycopene beadlet feeding significantly decreased the liver weight as a percent body weight in both CMO-II KO (lycopene 3.45 ± 0.09 % body weight; placebo 3.87 ± 0.09 % body weight and WT mice (lycopene 3.35 ± 0.09 % body weight compared to placebo 3.45 ± 0.09 % body weight; $P 0.006$) (data not shown).

KO mice have reduced reproductive organ weights

In both the 30 day and 4 day study, CMO-I KO mice and CMO-II KO mice had significantly smaller uteri as a percent of body weight compared to WT mice (**Tables 8&9**). Also

in the 30 day study, CMO-I KO mice had significantly smaller ovaries as a percent of body weight compared to WT mice but there was no modulation of diet on these weights (**Table 9**).

KO mice have greater accumulation of hepatic lipids

We analyzed lipid accumulation in the livers of our 30 day and 4 day-fed animals. For the most part, CMO-I KO mice and CMO-II KO mice accumulated more hepatic lipids compared to the appropriate WT group (**Tables 10, 11, 12, 13**). CMO-I KO mice fed AIN-93 G control diet and tomato powder were did not different from WT mice in liver lipid accumulation.

CMO-I KO mice have an increased incidence of minimal hepatic lipidosis

We analyzed the H&E stained liver slides from the 4 day study. Only CMO-I KO mice had signs of minimal hepatic lipidosis in 2 of the 8 representative slides (**Figure 9**). There were no differences between dietary groups.

CMO-II KO mice have altered cholesterol accumulation

We measured liver and serum total cholesterol accumulation in our 30 day and 4 day-fed animals. There was no difference in hepatic cholesterol due to diet or genotype in our 30 day-fed animals. In the 4 day feeding study, CMO-II KO mice fed placebo beadlet and lycopene beadlet diets had higher levels of hepatic total cholesterol compared to WT mice (**Table 14**).

There were significant differences in serum total cholesterol in our animals. CMO-I KO mice had lower serum cholesterol levels than WT animals in the 30 day study animals fed either tomato powder or AIN-93G (**Table 15**). There was an interaction between the CMO-I KO genotype and lycopene beadlet feeding in the 4 day study in serum cholesterol (**Table 17**). CMO-II KO mice in the 30 day and 4 day feeding studies had significantly reduced levels of serum cholesterol compared to WT mice (**Tables 16&18**).

Tomato powder-feeding modulates expression of PPAR γ and its target gene

We measured mRNA expression of PPAR γ and its target gene in the adipose tissue, *FABP4*, in the 30 day feeding animals. Tomato powder increased hepatic PPAR γ expression in both WT and CMO-II KO mice (diet effect P-value 0.006) (**Figure 10a**). In contrast, in the CMO-II KO mice fed placebo beadlet and lycopene beadlet diets, there was a decrease in hepatic PPAR γ mRNA expression compared to WT mice fed these diets (genotype effect P-value 0.046) (**Figure 10b**). In the CMO-I KO mouse livers there was a trend of tomato powder increasing PPAR γ mRNA expression (data not shown). In the adipose tissue, the CMO-II KO mice had increased PPAR γ expression compared to WT mice independent of diet (genotype effect P-value <0.001 for both groups) (**Figures 11a&11b**). Lycopene was shown to increase PPAR γ mRNA expression in WT mice and decrease its expression in CMO-I KO mice (diet effect P-value 0.023) (**Figure 12**). We also measured *FABP4* in the adipose tissue of these animals and found that CMO-II KO mice had decreased *FABP4* mRNA expression when fed AIN-93G and tomato powder diets (**Figure 13**). Lycopene decreased *FABP4* mRNA expression in CMO-I KO mice and WT mice (**Figure 14**).

Tomato powder-feeding increases PPAR α expression in CMO-I KO mice

We measured PPAR α mRNA expression in the livers of our 30 day feeding mice. Tomato powder increased PPAR α mRNA expression in both CMO-I KO and WT mice (**Figure 15**). There was a trend of tomato powder increasing PPAR α mRNA in CMO-II KO mice (data not shown).

Discussion

Carotenoid consumption has been associated with the reduction of many chronic diseases such as heart disease (55) and cancers of the lung, breast, prostate, cervix and ovary, to name a

few (2). Provitamin A carotenoid consumption helps to alleviate vitamin A deficiency symptoms which can lead to night blindness, impaired immune function, and numerous other negative health outcomes (1). Mice lacking CMO-I and CMO-II, enzymes responsible for cleaving carotenoids centrally (4) and eccentrically, respectively (8), have been used as models to investigate carotenoids and their metabolites. These knock-out mouse models show that there are differences in tissue accumulation of carotenoids and their metabolites in these two genotypes (23, 24).

We were interested in investigating different measures of lipid status such as hepatic lipid deposition and lipid metabolism-related genes in our CMO-I KO and CMO-II KO mice. CMO-I KO mice have been found to have elevated levels of hepatic lipids, serum free fatty acids and triglycerides, and genes involved in lipid metabolism regardless of dietary carotenoids, indicating these mice have a disruption in lipid metabolism (47). It has also been suggested that development of hepatic steatosis, a decrease in the expression of genes involved in fatty acid metabolism and an increase in triglyceride concentrations may be related to a lack in CMO-I (1, 47). We wanted to investigate lipid metabolism in older female mice because lipid alterations appeared to be more pronounced in the female mice (47). We also wanted to determine if tomato powder or lycopene modulated these changes. We fed 25-29 week old CMO-I KO mice, CMO-II KO mice and WT mice carotenoid-containing diets and their respective controls for either 30 or 4 days. We also investigated if a short term (4 day) carotenoid feeding would demonstrate similar changes in lipid deposition and gene regulation compared to a longer 30 day feeding in our mice.

Our lab has previously shown the carotenoid distribution of lycopene and tomato powder-fed CMO-I KO and CMO-II KO male mice (23) and lycopene and β -carotene-fed CMO-I KO

male and female mice (24). As expected, in our current study we found that female CMO-I KO mice compared to WT mice, accumulated significantly lower levels of lycopene from lycopene beadlet diet and the tomato powder diet in their livers regardless of the duration of feeding. Our findings also support the role of CMO-II as a key enzyme in the metabolism of lycopene because we found that CMO-II KO mice fed either lycopene or tomato powder to have significantly higher levels of total lycopene in their livers and adipose compared to their respective WT controls. These results support previous evidence that CMO-I KO mice have upregulated expression of CMO-II in some tissues suggesting that there is a compensatory mechanism involved with these enzymes (23). To our surprise, we found that the serum lycopene accumulation did not follow the same patterns as the liver or the adipose tissue lycopene accumulation. CMO-I KO mice fed lycopene beadlet diet had enhanced serum accumulation of lycopene compared to their lycopene beadlet-fed WT controls. We did not measure lycopene concentrations in the serum of our 4 day animals. In a previous study from our lab, both male and female CMO-I KO mice were fed lycopene beadlet diet, there were no significant difference in serum total lycopene accumulation compared to WT lycopene beadlet diet-fed animals after 60 days of feeding (24). Other than the serum lycopene results from the current work, our laboratory has seen a consistently higher tissue accumulation of lycopene when either tomato powder or lycopene beadlet diet is fed to CMO-II KO mice and a lower distribution of lycopene in CMO-I KO mice (23,24).

In order to determine differences in body weight and organ weights we monitored animal weight gain throughout the studies and weighed the organs at the termination of the study. CMO-I KO mice were not different from WT mice in body weight at the end of either the 30 day or the 4 day studies. These findings contradict previous research which found CMO-I KO mice to be

heavier than WT mice (24, 47). The differences between our mice and the mice in these other studies were that our mice were not fed a high fat diet. This suggests that under a high fat diet (16.7% dry weight and 30% w/w) the CMO-I KO genotype is more vulnerable to enhanced lipid accumulation and weight gain than WT mice, but if the mice are kept on a lower fat (7-10% of dry weight) diet their body weights and tissue lipids will be more similar to WT mice. We hypothesized that our CMO-II KO mice would not be different in body weight at the completion of our feeding study because of previous work from our lab with male mice fed lower fat diets (23).

Conversely, we found our CMO-II KO mice weighed less than their respective WT mice at the completion of both the 30 day and 4 day studies. The mice in Ford study were younger (9-12 weeks old) male mice, whereas our mice were all older (30-35 weeks old) female mice (23). One conclusion from this data is that, when female CMO-I KO mice are fed a standard level of fat in their diets they do not differ from WT mice in body weight. Also, we conclude that female CMO-II KO mice are smaller than WT mice and that the presence of carotenoids in the diet does not overcome the difference.

Contrary to our body weight data, we found reproductive organ weights, as a percent of body weight, to differ by genotype which has been demonstrated, in part, in the literature. Previous findings from our lab found CMO-I KO mice to have smaller uteri, as a percent of body weight, regardless of diet compared to WT mice (24). We found both CMO-I KO mice and CMO-II KO mice uteri to be smaller than their respective WT controls. This is of interest to us because we found the reproductive capacity of our breeding colonies of CMO-II KO mice to be decreased (average~2 pups/litter) compared to CMO-I KO mice (average~5 pups/litter) fed a standard chow diet. Although CMO-I KO mice did have increased litter sizes compared to

CMO-II KO mice, the litter sizes are still smaller compared to WT mice (mean litter size 6.2 according to Jackson Laboratory).

In addition to smaller uteri weights, we found that the ovary weights, as a percent of body weight, were lower in the 30 day CMO-I KO mice study animals compared to WT mice.

Considering this novel finding of decreased ovary weights in CMO-I KO mice, and the decreased uteri weight data in both CMO-I KO and CMO-II KO mice, it would be interesting to investigate the hormonal differences between these mice and WT mice in order to determine if the reproductive differences in these genotypes were due to altered hormone balances.

In the 30 day study, CMO-II KO mice had significantly larger livers, spleens and kidneys as a percent body weight compared to WT mice. These results were not generally seen in the 4 day study. Additionally, in the 30 day study dietary lycopene resulted in decreased liver weights in both CMO-II KO mice and WT mice. No dietary effects were seen in any other tissues in either of the studies. In the 4 day study kidney weights, as percent body weight, were significantly lower in CMO-I KO mice compared to WT mice. The differences in organ weights, as percent body weight, were generally independent of carotenoid feeding and demonstrate the genotypic differences between the two strains of KO mice compared to WT mice. This further supports the idea that carotenoid cleavage enzymes may have a number of roles in tissues beyond those related to carotenoid metabolism.

We next wanted to determine how tomato powder and lycopene beadlet diets might influence lipid accumulation in the livers of our KO mice. We hypothesized that our CMO-I KO mice would develop hepatic steatosis independently of diet, in line with the results reported by Hessel et. al., who found that CMO-I KO mice developed fatty liver regardless of vitamin A status of the diet (47). Our findings fell in line with these results, but hepatic lipid alterations

were more modest. Female CMO-I KO mice had a greater accumulation of hepatic lipids compared to their respective WT controls. However, when histology was done on representative liver samples, we saw that 75% of livers were normal and 25% of the samples in the CMO-I KO mouse group only showed minimal hepatic lipidosis. One of the 2 livers that showed signs of minimal hepatic lipidosis was from a carotenoid-fed animal and the other was from a control diet-fed animal. These findings support the idea that carotenoids in the diet do not significantly impact lipid deposition and steatosis in the livers of CMO-I KO mice fed lower fat diets.

Interestingly, the same group that reported hepatic steatosis in their CMO-I KO mice also reported hepatic steatosis in CMO-II KO mice fed lower fat diets supplemented with zeaxanthan (10). Male and female CMO-II KO mice, heterozygous and homozygous CMO-II KO mice fed zeaxanthan, but not chow-fed homozygous CMO-II KO mice. In our CMO-II KO mice we also found a modest increase in hepatic lipid deposition, but H&E staining did not reveal evidence of hepatic steatosis. Also, our CMO-II KO mice accumulated hepatic lipids irrespective of carotenoid-containing diets. One explanation for the differences of current findings with those of Amengual et al., is that the CMO-II KO mice in our study received lycopene (0.1g lycopene/kg diet), not zeaxanthan. The mice in the Amengual et al. report were also both male and female, 13 weeks old at sacrifice, and fed a 0.05 mg/g zeaxanthan supplemented vitamin A-deficient (vitamin A levels not provided) experimental diets for 8 weeks. Of greater importance may be that Amengual and coworkers (10) fed a vitamin A-deficient diet for 8 weeks which would render these animals close to outright vitamin A deficiency.

Our lab previously showed that CMO-I KO mice fed high fat and cholesterol-containing diets with lycopene had significantly lower serum cholesterol levels when compared to CMO-I KO mice fed cholesterol diet without lycopene (24). Palozza et al. reported that when lycopene

was administered to a variety of human cells in culture; there was a dose dependent decrease in total intracellular cholesterol content *in vitro* along with a reduction of the expression of HMG-CoA reductase (56). Based on these experiments we wanted to investigate both the serum and hepatic total cholesterol levels in our carotenoid cleavage enzyme knock-out mice. There were no dietary effects on hepatic cholesterol accumulation in our mice but we did find that when CMO-II KO mice were fed placebo and lycopene beadlet diets they had increased total cholesterol levels. In the 30 day feeding study CMO-I KO and CMO-II KO mice fed AIN-93G and tomato powder diets had lower levels of serum cholesterol compared to WT mice. CMO-II KO mice also had lower levels of serum cholesterol in the 4 day study. There was also a dietary effect in the 30 day study that showed an increase in serum cholesterol in WT mice with lycopene feeding but a decrease in CMO-II KO mouse serum cholesterol.

In order to determine potential effects of tomato powder and lycopene feeding on genes involved in lipid metabolism we performed RT-PCR on our 30 day liver and adipose samples. We were specifically interested in the PPARs, especially PPAR- γ because PPAR γ and RXR α agonists increased the rate of gene expression of CMO-I by forming a heterodimer in the promoter region of CMO-I thus increasing transcription of the CMO-I gene (41). Also, the peroxisome proliferator response element (PPRE) site is located in the CMO-I promoter. This information is important for understanding the role of this carotenoid cleavage enzyme in lipid metabolism.

In our study we found that tomato powder feeding increased hepatic PPAR γ mRNA expression in the CMO-II KO and WT mice and there was a trend for an increase expression in CMO-I KO mice. These effects caused by tomato powder were not seen in the adipose tissue. In addition, we found PPAR γ expression to be increased in the adipose of CMO-II KO mice, but to

our surprise, FABP4, a PPAR γ target gene in the adipose, was decreased in these same mice. We have no explanations for these findings.

Another interesting finding from our mRNA expression data was that dietary lycopene modulated PPAR γ mRNA expression differently compared tomato powder feeding. Feeding a lycopene beadlet diet decreased mRNA expression of both PPAR γ and FABP4 in the adipose of CMO-I KO mice. These results are similar to those in a rat study from our lab, where they found that lycopene beadlet diets decreased mRNA expression of PPAR γ and its target gene FABP3 in the rat adrenals and kidneys (46). It was hypothesized that CMO-II activity on lycopene may be what is causing this decrease in expression of PPAR γ and CMO-I (47). This hypothesis is based on data that showed that a CMO-II short chain cleavage product of β -carotene, apocarotenal 14 (apo14), repressed known PPAR γ , PPAR α and RXR α transcriptional responses by their respective agonists (57). It is therefore possible that asymmetric cleavage of lycopene would also produce lycopinals or their metabolic products that could repress PPAR γ -regulated target genes, such as FABP4. Additionally, it is possible that other cleavage products of carotenoids, and not just asymmetric cleavage products, could have regulatory effects on PPARs and RXR α transcriptional responses. This hypothesis could provide some explanation as to why FABP4 was decreased in the adipose tissue of multiple carotenoid-containing (PE, PF and β -carotene) tomato powder-fed CMO-II KO mice (57).

We were also interested in looking at PPAR α and its target gene ACOX1. We found that tomato powder significantly increased the mRNA expression of PPAR α in the livers of CMO-I KO mice. Hessel et al. fed a vitamin A sufficient standard diet to both male and female mice and did not find any differences in liver mRNA expression of PPAR α between CMO-I KO and WT (47). In contrast, these researchers found hepatic ACOX1 mRNA expression to be upregulated in

CMO-I KO mice compared to WT mice. We did not see any differences in either hepatic or adipose mRNA expression of ACOX1. As seen in the PPAR γ and FABP4 results from the adipose of CMO-I KO mice, the mRNA expression of the regulator gene does not always influence the mRNA expression of the target gene. Protein levels of PPAR α and ACOX1 should also be analyzed in order to determine if levels of these proteins can be influenced by tomato powder. If the protein levels of ACOX1 are seen to be decreased, as seen in Hessel et al. (47), investigation into apo14 levels in the liver would be interesting to measure.

The data presented provide a description of alterations in lipid deposition and organ weights in female CMO-I KO mice and CMO-II KO mice. These results suggest that the lack of a carotenoid cleavage enzyme in itself has more influence on altering lipids than the addition of tomato carotenoids in the diet. In addition, the data on carotenoid accumulation in female CMO-II KO mice fed lycopene and tomato powder-containing diets is novel and has not been published previously and it is important to understand how these animals accumulate these carotenoids.

Acknowledgements

We acknowledge the IGB animal facility staff and veterinarians for their help with maintaining the health of our animals. Additionally, we thank the Veterinary Medicine histology center staff for their help with staining our liver samples and Dr. Wallig for his help with analyzing our histology slides. We thank our past undergraduate research assistants, especially CJ Bauer, for their help throughout the study.

Figures and Tables

Figures

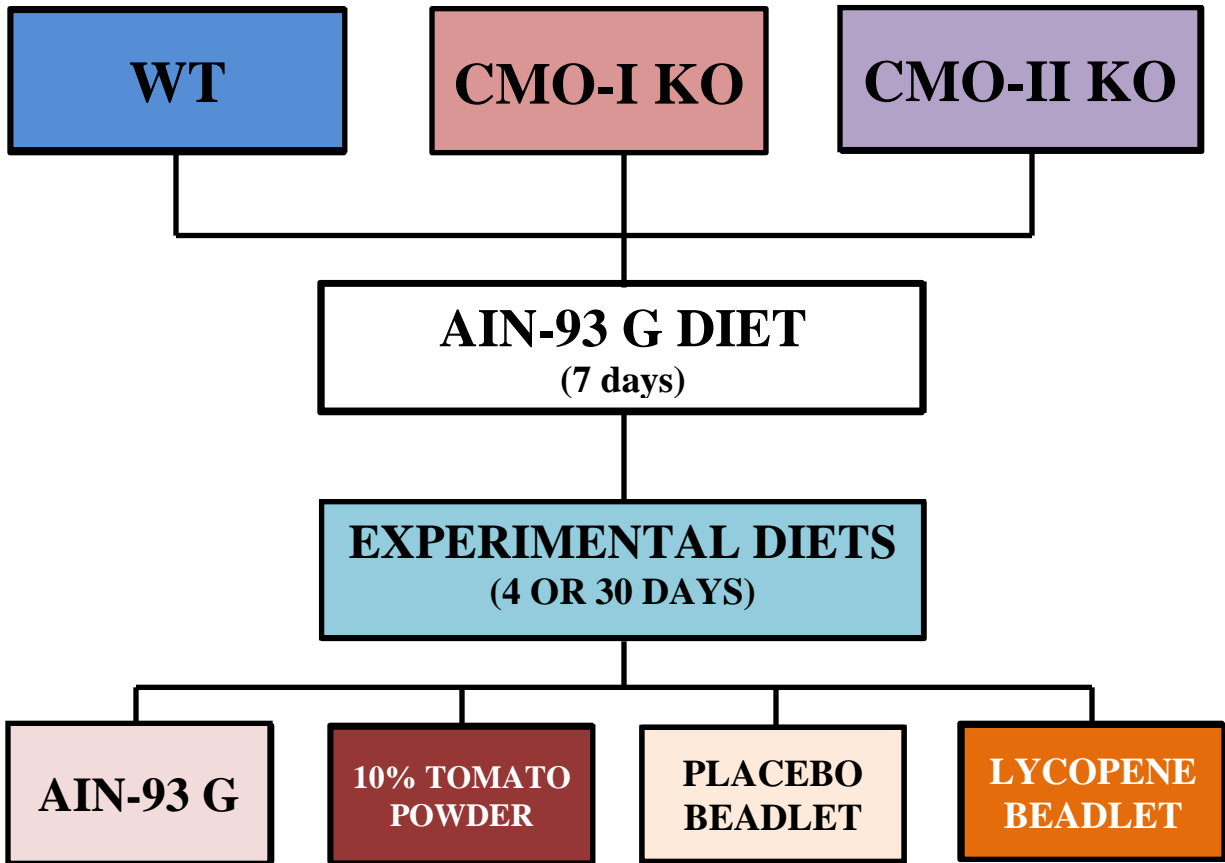


Figure 1: Study Design. n=10/genotype/diet/study; WT (C57BL/6Jx129x1/SvJ)

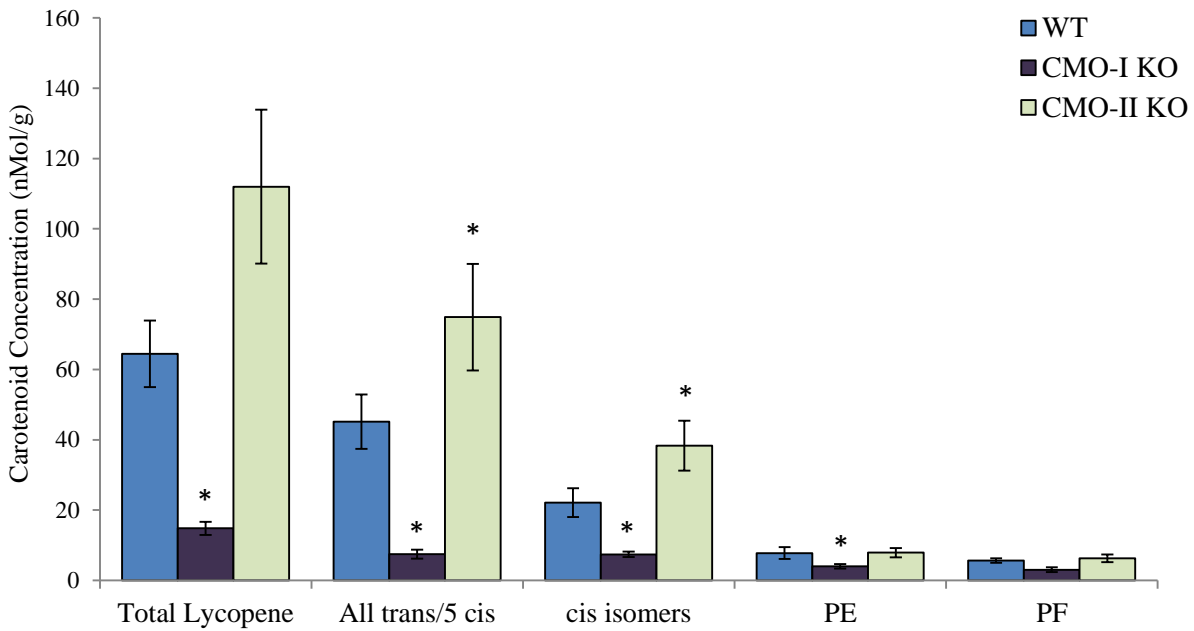


Figure 2: Carotenoid accumulation in the livers of tomato powder-fed 30 day study animals;
 * Indicates significantly different from WT; n=6-10/genotype

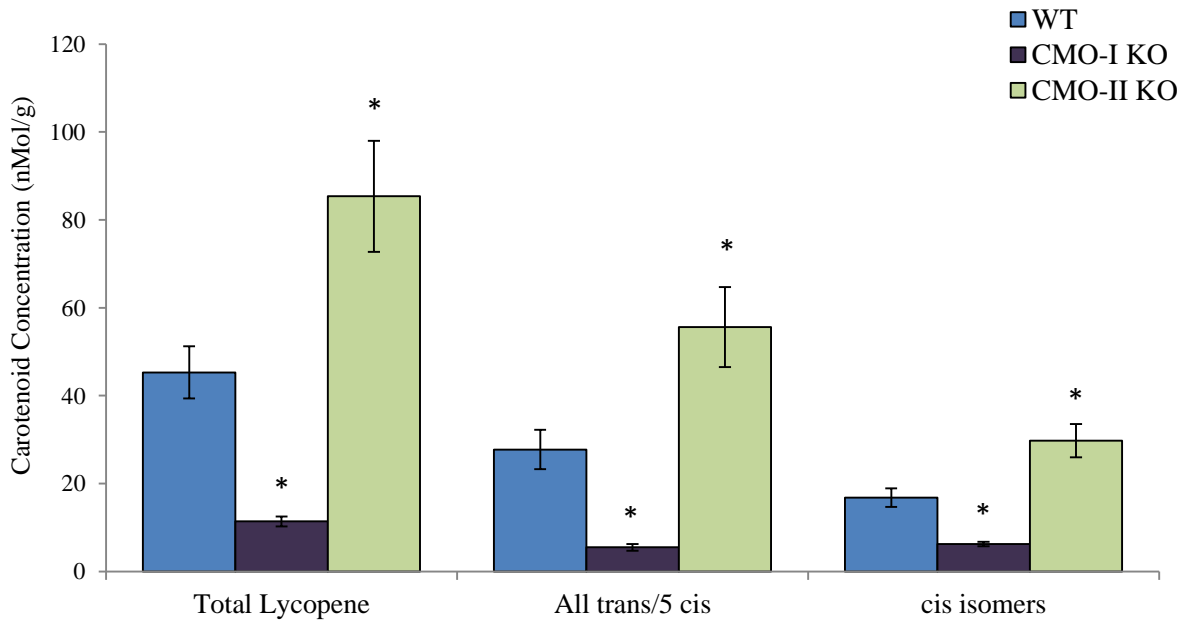


Figure 3: Carotenoid accumulation in the livers of lycopene beadlet-fed 30 day study animals;
 * Indicates significantly different from WT; n=6-10/genotype

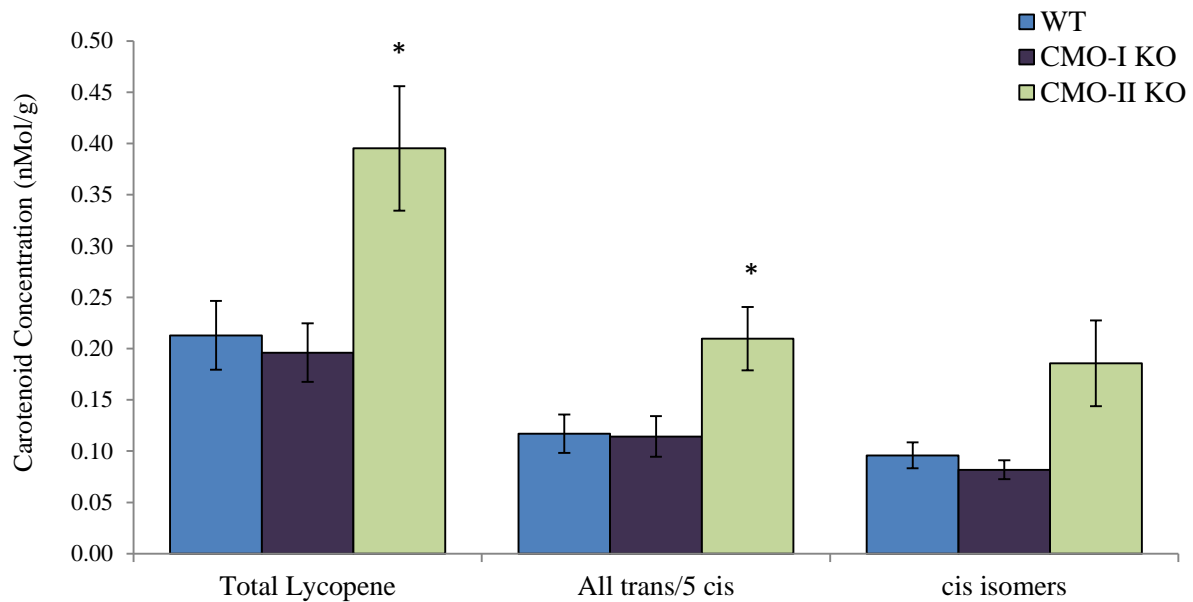


Figure 4: Carotenoid accumulation in the gonadal adipose of tomato powder-fed 30 day study animals; * Indicates significantly different from WT; n=8/genotype

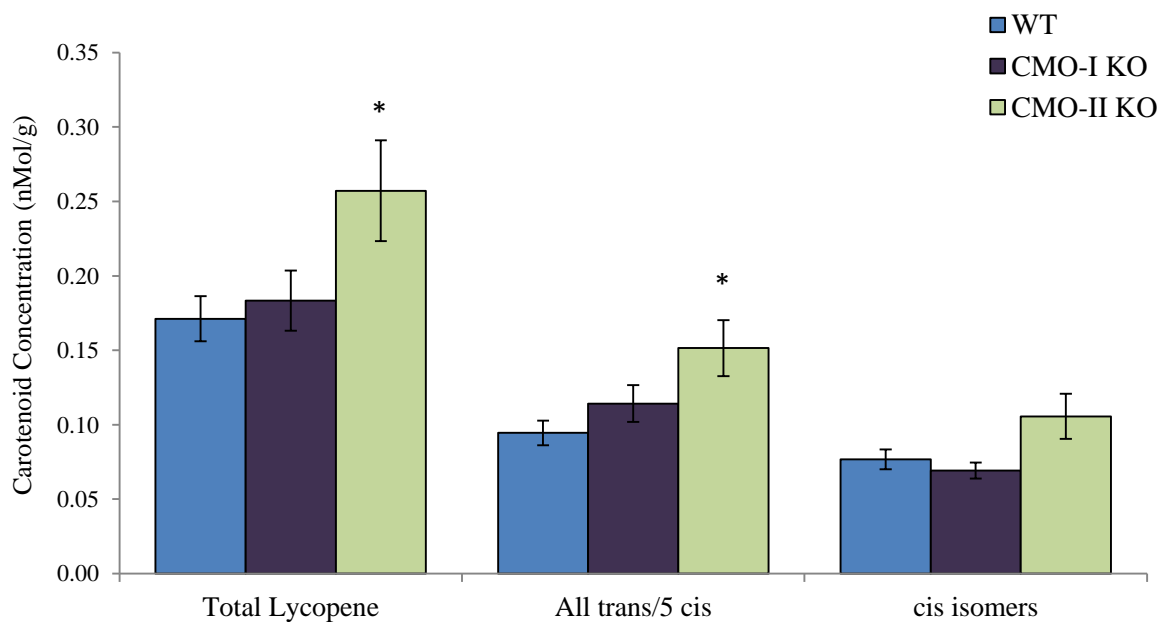


Figure 5: Carotenoid accumulation in the gonadal adipose of lycopene beadlet-fed 30 day study animals; * Indicates significantly different from WT; n=7-8/genotype

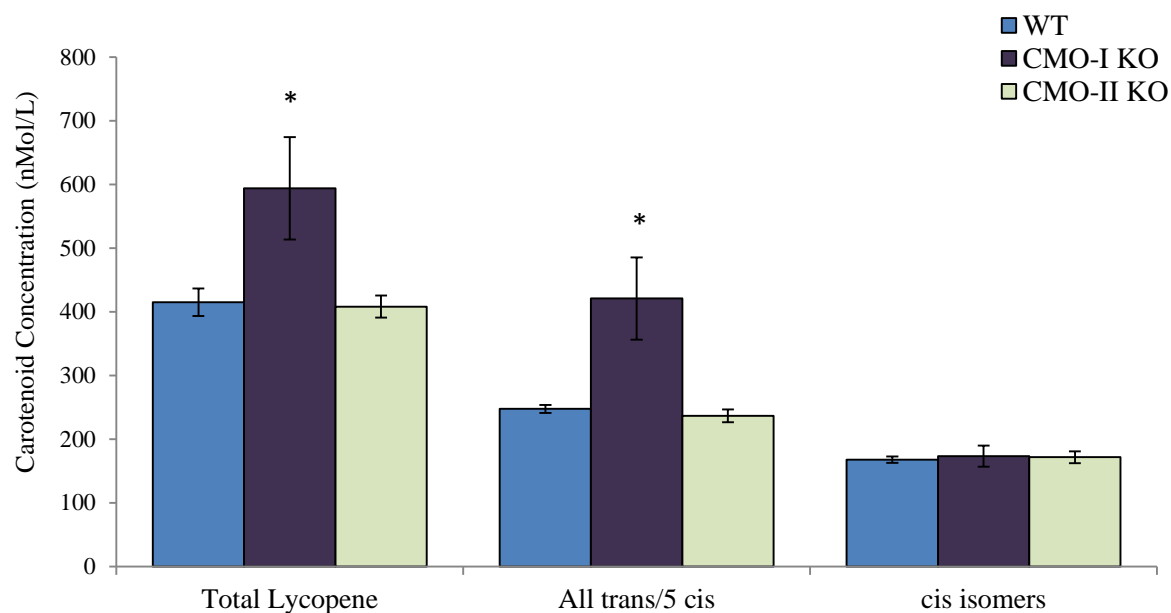


Figure 6: Carotenoid accumulation in the serum of lycopene beadlet-fed 30 day study animals;
 * Indicates significantly different from WT; n=5-6/genotype

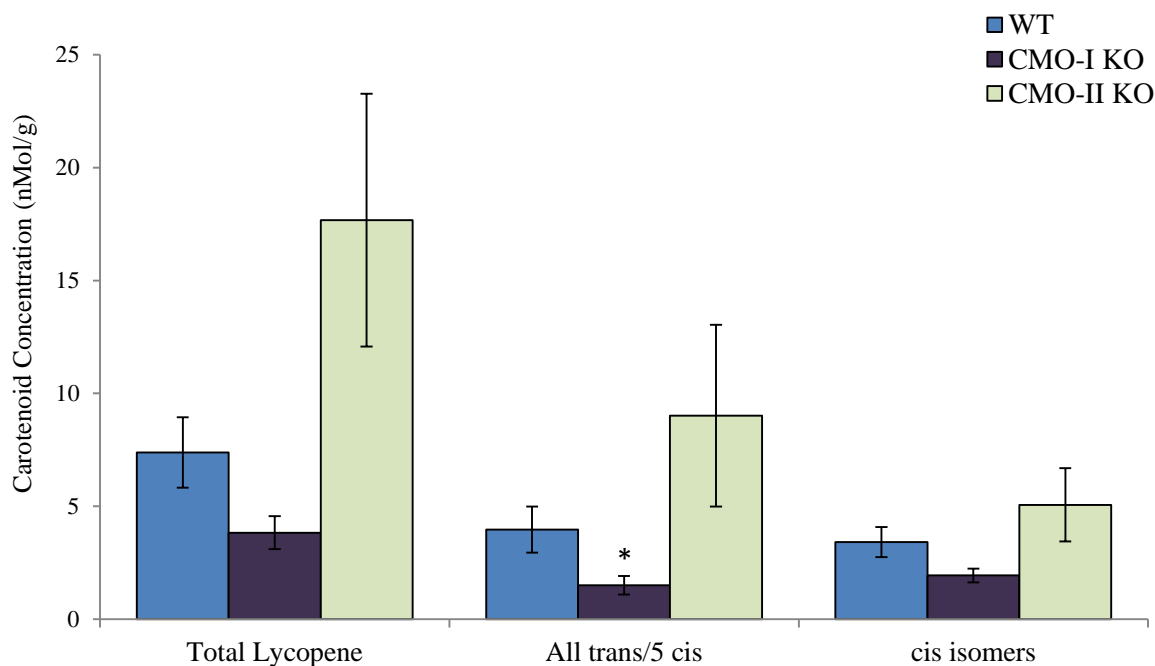
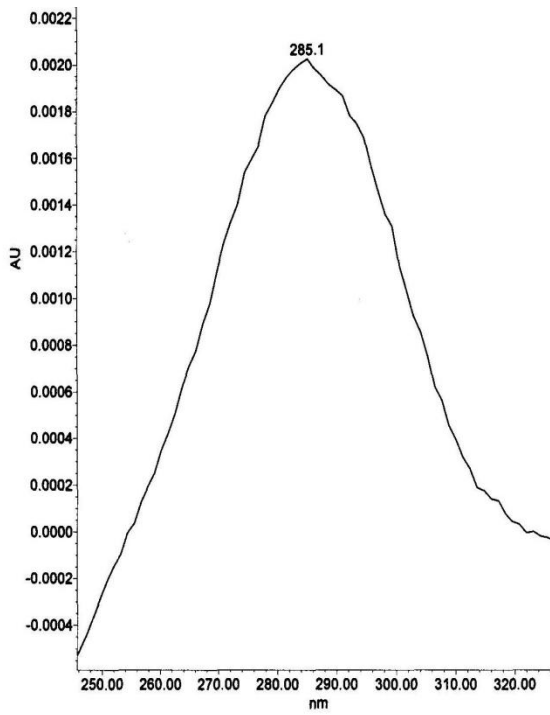
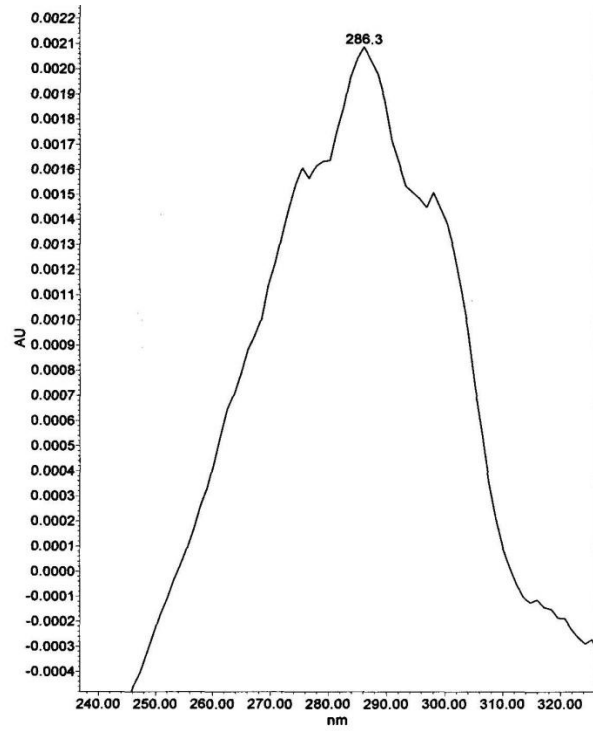


Figure 7: Carotenoid accumulation in the liver of lycopene beadlet-fed 4 day study animals;
 *Indicates significantly different from WT; n=6-7/genotype

A.



B.



C.

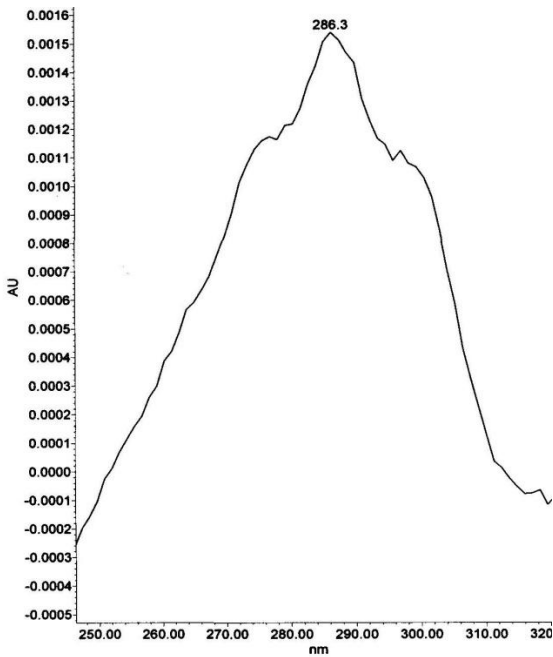
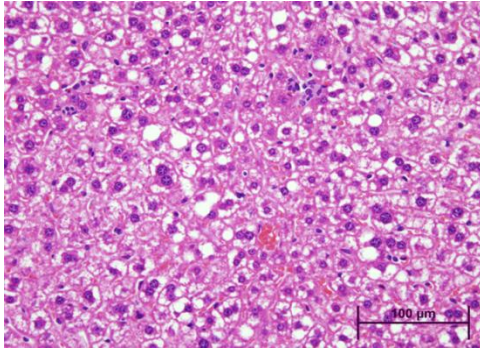
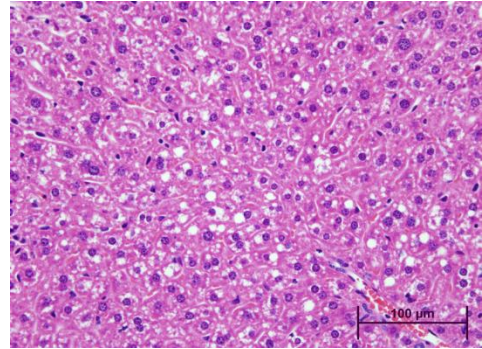


Figure 8: Comparison of PE and pseudo PE spectra. A) Adipose pseudo PE peak in CMO-II KO mouse fed tomato powder for 4 days B) Standard PE peak C) Liver PE peak in a CMO-II KO mouse fed tomato powder for 30 days

A.



B.



C.

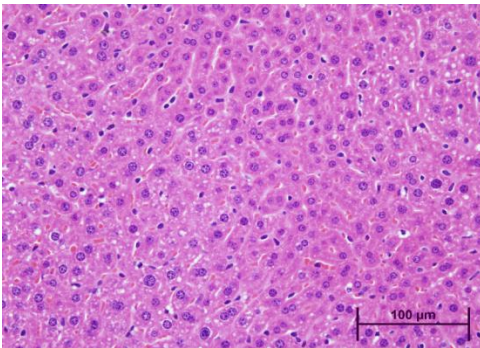


Figure 9: H&E stained CMO-I KO liver slides compared to a WT slide. A) CMO-I KO mouse fed tomato powder for 4 days, minimal lipidosis B) CMO-I KO mouse fed placebo beadlet diet for 4 days, minimal lipidosis C) WT mouse fed AIN-93G diet for 11 days, normal liver

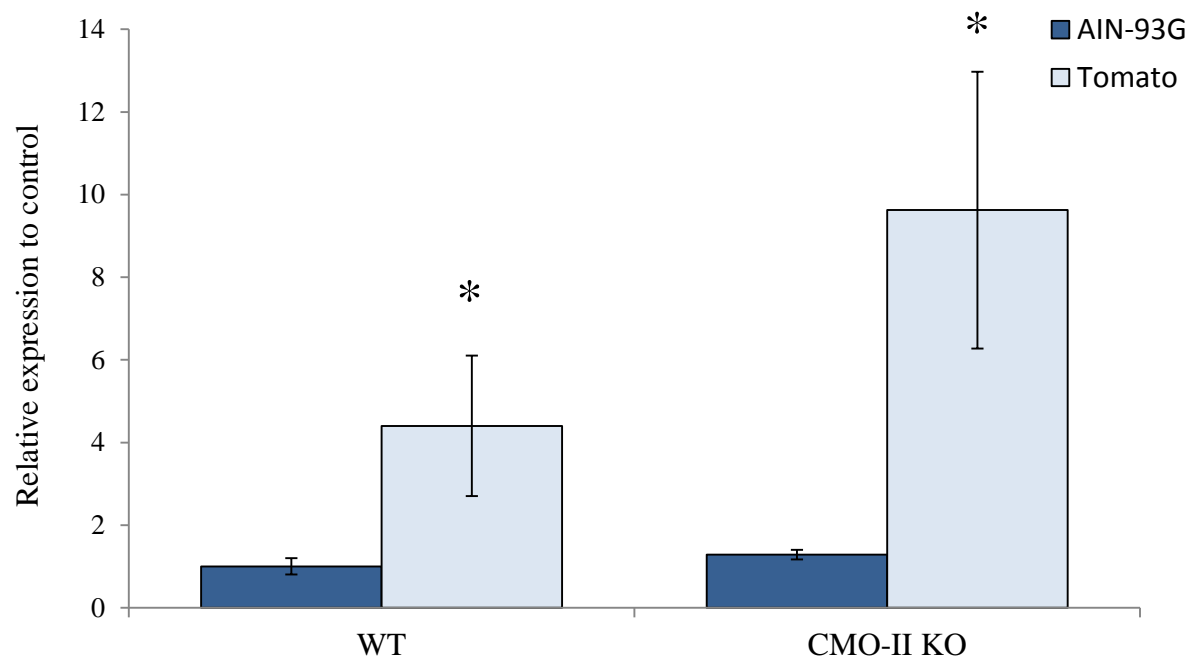


Figure 10a: mRNA expression of PPAR γ in the liver of CMO-II KO and WT mice; * Indicates significantly different from control diet; n=4-8/genotype/diet

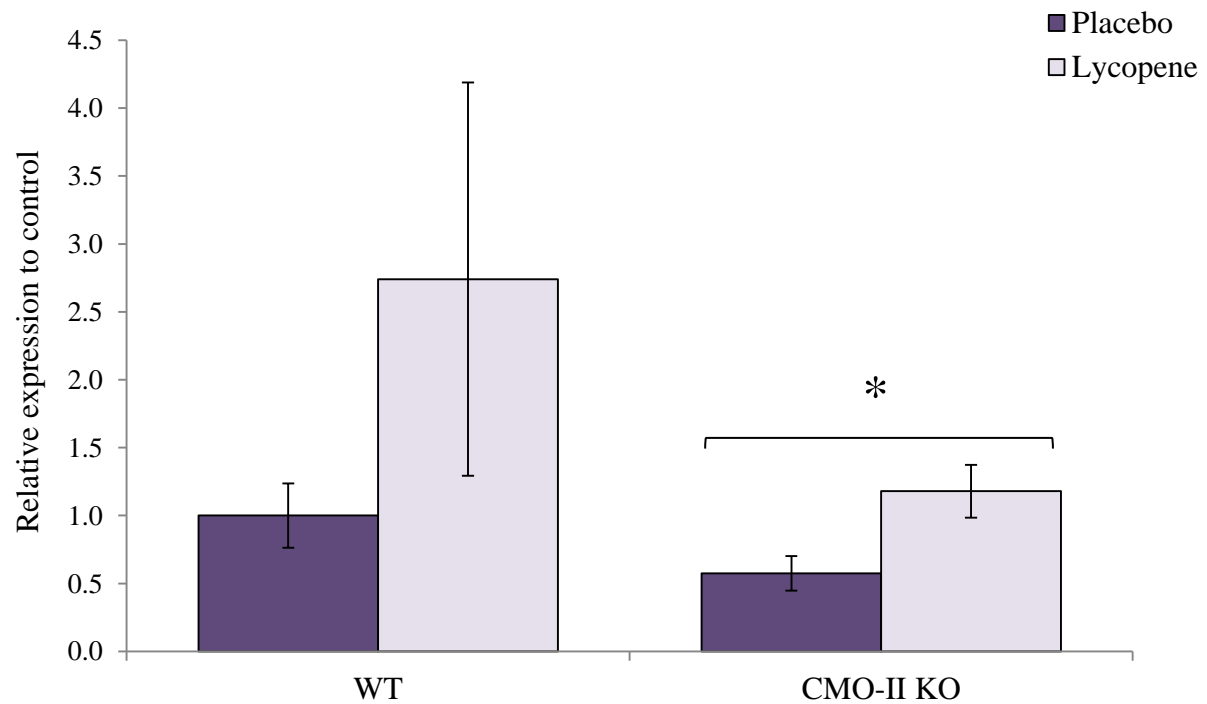


Figure 10b: mRNA expression of PPAR γ in the liver of CMO-II KO and WT mice; * Indicates significantly different from WT; n=5-6/genotype/diet

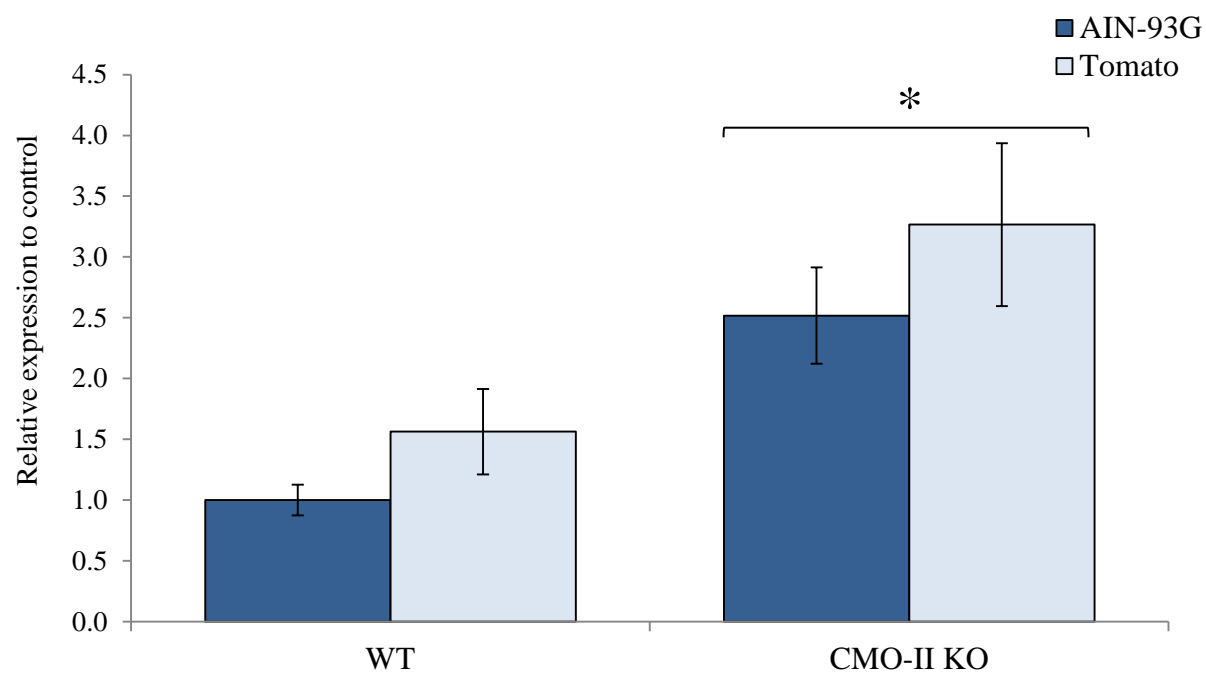


Figure 11a: mRNA expression of PPAR γ in the adipose tissue of CMO-II KO and WT mice; * Indicates significantly different from WT; n=7-9/genotype/diet

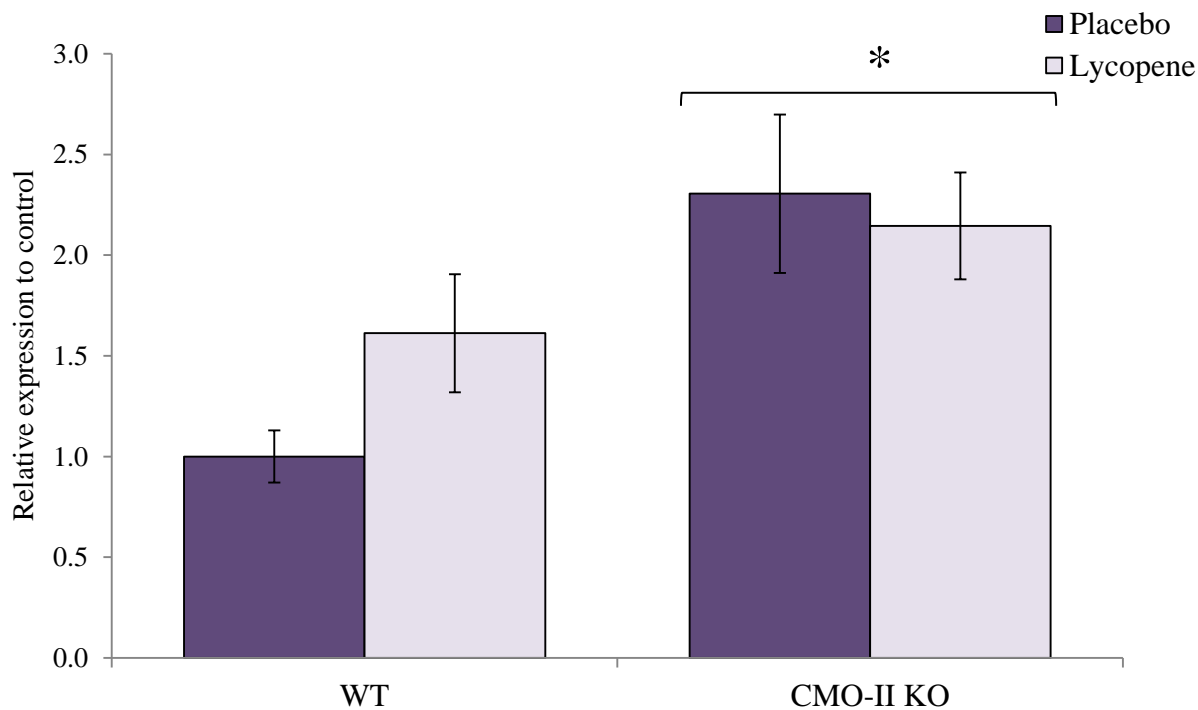


Figure 11b: mRNA expression of PPAR γ in the adipose tissue of CMO-II KO and WT mice; * Indicates significantly different from WT; n=6-9/genotype/diet

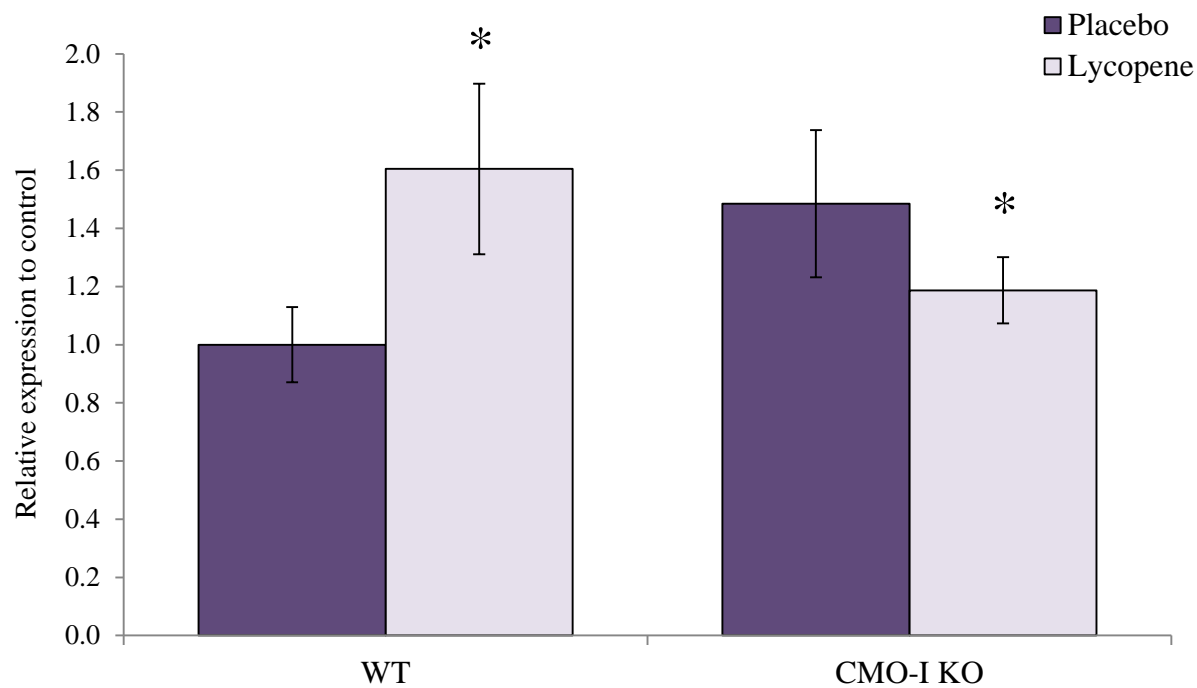


Figure 12: mRNA expression of PPAR γ in the adipose tissue of CMO-I KO and WT mice; * Indicates significantly different from control diet; n=8-9/genotype/diet

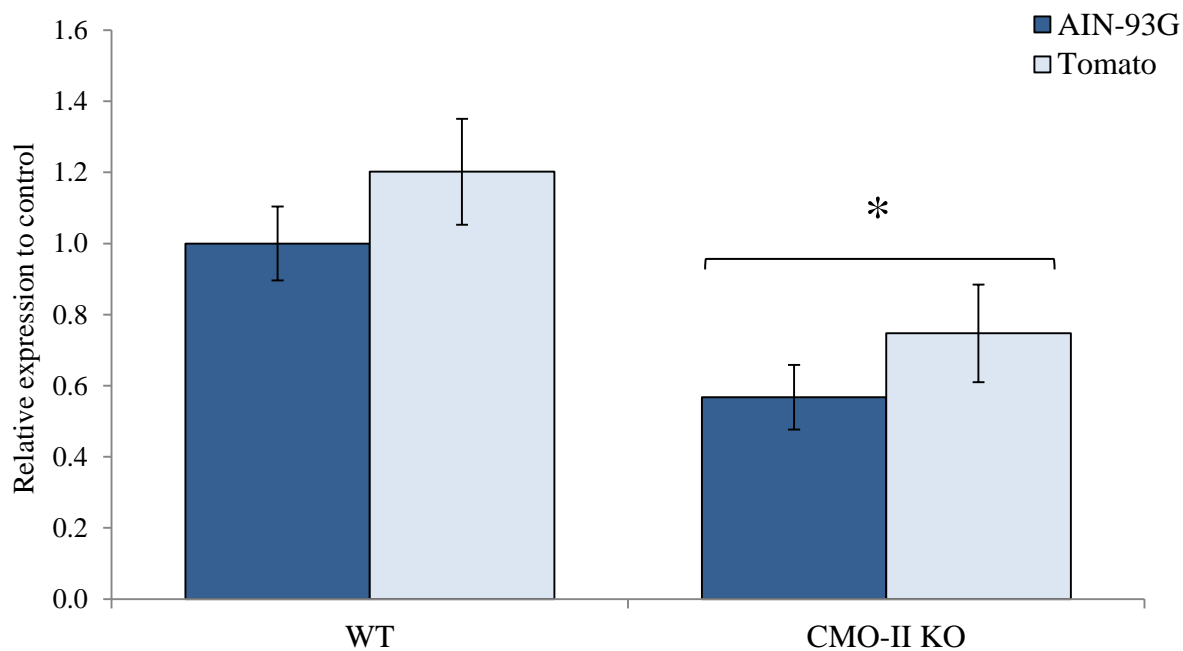


Figure 13: mRNA expression of FABP4 in the adipose tissue of CMO-II KO and WT mice; * Indicates significantly different from WT; n=7-9/genotype/diet

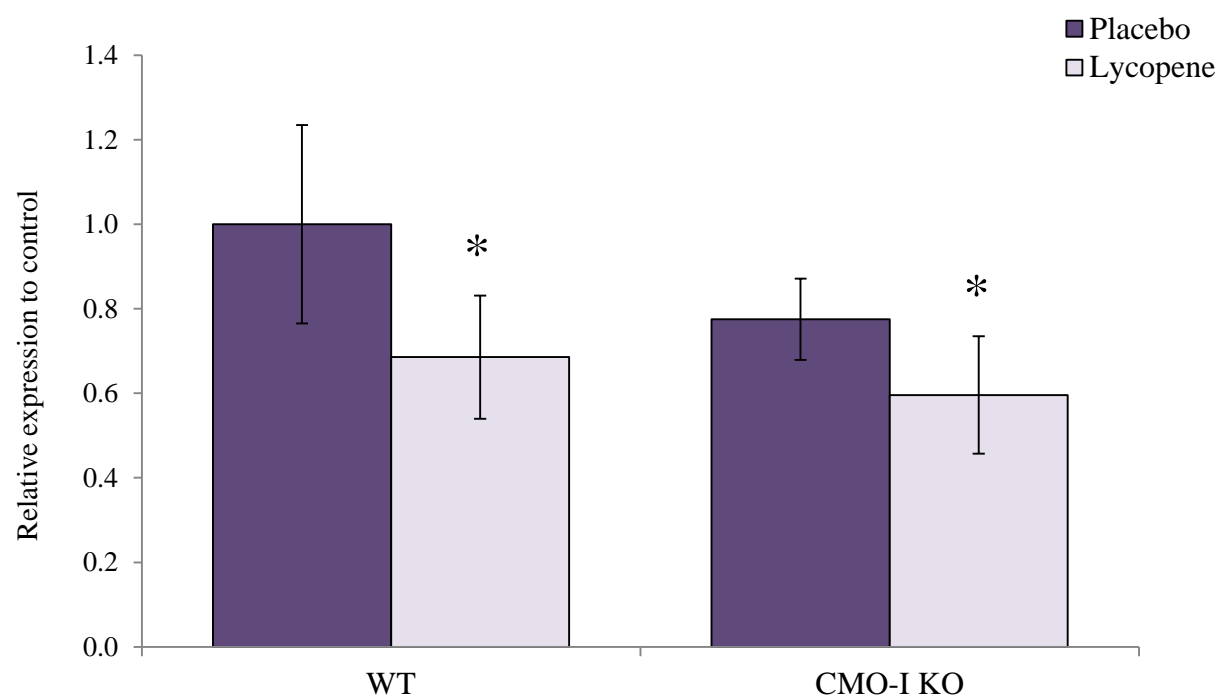


Figure 14: mRNA expression of FABP4 in the adipose tissue of CMO-I KO and WT mice; * Indicates significantly different from control diet; n=7-9/genotype/diet

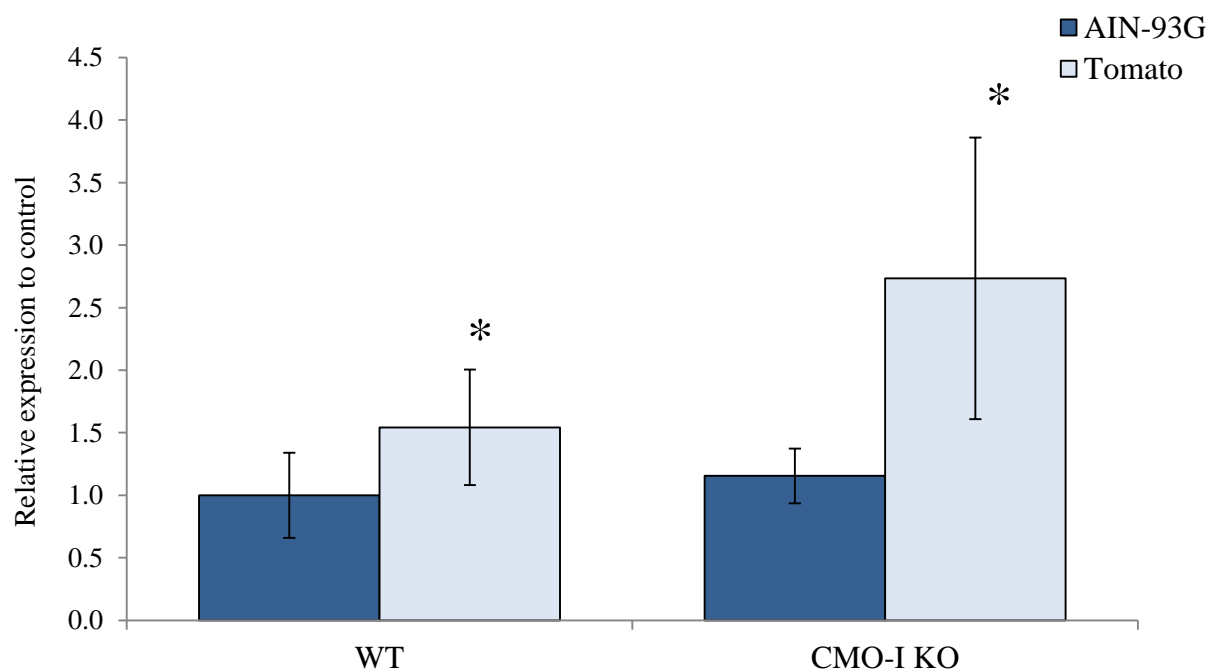


Figure 15: mRNA expression of PPAR α in the liver of CMO-I KO and WT mice; * Indicates significantly different from control diet; n=7-9/genotype/diet

Tables

Table 1: Diet composition of 30 day and 4 day studies.

	AIN-93G	10% Tomato Powder ^{A,B}	Placebo Beadlet	10% Lycopene Beadlet ^{C,D}
Ingredients	(g/kg diet)			
Cornstarch	397.5	331.5	397.5	397.5
Casein	200	187.5	200	200
Maltodextrin	102	132	102	102
Sucrose	100	100	100	100
Fiber	50	34	50	50
Mineral Mix	35	35	35	35
Vitamin Mix (Low Vitamin A)	10	10	10	10
L-cystine	3	3	3	3
Choline Bitartrate	2.5	2.5	2.5	2.5
Soybean Oil	100	65	100	100
Tomato Powder	0	100	0	0
Lycopene Beadlets	0	0	0	1.0
Placebo Beadlets	0	0	1.0	0

^A The 30 day study contained: 40 ± 5 nMol/g diet *cis* isomer lycopene; 140 ± 22 nMol/g diet all-*trans*/5-*cis* lycopene; 8 ± 1.3 nMol/g diet of phytoene (PE); 6 ± 0.9 nMol/g diet phytofluene (PF); 2 ± 0.3 nMol/g β -carotene (BC) ^B The 4 day study contained: 46 ± 0.6 nMol/g diet *cis* isomer lycopene; 188 ± 12 nMol/g diet all-*trans*/5-*cis* lycopene; 12 ± 0.02 nMol/g diet of PE; 7 ± 0.7 nMol/g diet PF; 2 ± 0.1 nMol/g diet BC ^C The 30 day study contained 22 ± 2 nMol/g diet *cis* isomer lycopene; 129 ± 4.7 nMol/g diet all-*trans*/5-*cis* lycopene ^D The 4 day study contained 28 ± 2 nMol/g diet *cis* isomer lycopene; 148 ± 19 nMol/g diet all-*trans*/5-*cis* lycopene

Table 2: Carotenoid accumulation in the livers of tomato powder-fed 30 day study animals.

Carotenoid	WT	CMO-I KO	CMO-II KO	P-value**	
	nMol/g weight \pm SEM			CMO-I KO	CMO-II KO
Total lycopene	64.5 \pm 9.5	14.8 \pm 1.9*	112 \pm 22.9	<0.001	0.41
All trans/5cis	45.2 \pm 7.7	7.5 \pm 1.3*	75.0 \pm 15.2*	0.002	0.04
Cis isomers	22.1 \pm 2.2	7.4 \pm 0.8*	38.3 \pm 7.1*	<0.001	0.04
PE	7.8 \pm 1.7	7.9 \pm 1.3*	3.7 \pm 0.6	0.03	0.80
PF	5.6 \pm 0.7	3.0 \pm 0.7	6.3 \pm 1.1	0.28	0.21

* Indicates significantly different from WT; bolded P-value indicates significant of <0.05; n=6-10/genotype; **P-values represent comparison between WT mice and each KO mouse genotype individually

Table 3: Carotenoid accumulation in the livers of lycopene beadlet-fed 30 day study animals.

Carotenoid	WT	CMO-I KO	CMO-II KO	P-value**	
	nMol/g weight \pm SEM			CMO-I KO	CMO-II KO
Total lycopene	46.3 \pm 5.9	11.2 \pm 1.1*	85.4 \pm 12.6*	<0.001	0.02
All trans/ 5 cis	27.7 \pm 4.5	5.5 \pm 0.7*	55.6 \pm 9.1*	<0.001	0.02
cis isomers	16.8 \pm 2.1	6.3 \pm 0.5*	29.7 \pm 3.8*	<0.001	0.02

* Indicates significantly different from WT; bolded P-value indicates significant of <0.05; n=6-10/genotype; **P-values represent comparison between WT mice and each KO mouse genotype individually

Table 4: Carotenoid accumulation in the gonadal adipose of tomato powder-fed 30 day study animals.

Carotenoid	WT	CMO-I KO	CMO-II KO	P-value**	
	nMol/g weight \pm SEM			CMO-I KO	CMO-II KO
Total lycopene	0.21 \pm 0.03	0.20 \pm 0.03	0.40 \pm 0.06*	0.71	0.02
All-trans/ 5-cis	0.12 \pm 0.02	0.11 \pm 0.02	0.21 \pm 0.03*	0.92	0.02
cis isomers	0.10 \pm 0.02	0.08 \pm 0.01	0.19 \pm 0.04	0.51	0.07

* Indicates significantly different from WT; bolded P-value indicates significant of <0.05; n=8/genotype; **P-values represent comparison between WT mice and each KO mouse genotype individually

Table 5: Carotenoid accumulation in the gonadal adipose of lycopene beadlet-fed 30 day study animals.

Carotenoid	WT	CMO-I KO	CMO-II KO	P-value**	
	nMol/g weight \pm SEM			CMO-I KO	CMO-II KO
Total lycopene	0.17 \pm 0.02	0.18 \pm 0.02	0.26 \pm 0.03*	0.886	0.03
All trans/ 5 cis	0.11 \pm 0.01	0.09 \pm 0.01	0.15 \pm 0.02*	0.226	0.01
cis isomers	0.08 \pm 0.01	0.07 \pm 0.01	0.11 \pm 0.02	0.446	0.10

* Indicates significantly different from WT; bolded P-value indicates significant of <0.05; n=7-8/genotype; **P-values represent comparison between WT mice and each KO mouse genotype individually

Table 6: Carotenoid accumulation in the serum of lycopene beadlet-fed 30 day study animals.

Carotenoid	WT	CMO-I KO	CMO-II KO	P-value**	
	nMol/L weight \pm SEM			CMO-I KO	CMO-II KO
Total lycopene	415 \pm 21.7	594 \pm 80.4*	408 \pm 17.2	0.04	0.81
All-trans/5-cis	247 \pm 21.0	421 \pm 64.6*	237 \pm 10.0	0.02	0.65
cis isomers	168 \pm 5.2	173 \pm 16.4	172 \pm 9.3	0.74	0.72

* Indicates significantly different from WT; bolded P-value indicates significant of <0.05; n=5-6/genotype; **P-values represent comparison between WT mice and each KO mouse genotype individually

Table 7: Carotenoid accumulation in the liver of lycopene beadlet-fed 4 day study animals.

Carotenoid	WT	CMO-I KO	CMO-II KO	P-value**	
	nMol/g weight \pm SEM			CMO-I KO	CMO-II KO
Total lycopene	7.4 \pm 1.6	3.8 \pm 1.9	17.7 \pm 5.6	0.17	0.22
All trans/5 cis	3.8 \pm 1.0	1.5 \pm 0.4*	9.0 \pm 4.0	0.02	0.10
cis isomers	3.4 \pm 0.7	1.9 \pm 0.3	5.1 \pm 1.6	0.10	0.30

* Indicates significantly different from WT; bolded P-value indicates significant of <0.05; n=6-7/genotype; **P-values represent comparison between WT mice and each KO mouse genotype individually

Table 8: Body and organ weights of the 30 day feeding study.

Treatment	WT	CMO-I KO	CMO-II KO	P-value**	
				CMO-I KO	CMO-II KO
Body Weight (g ± SEM)					
TP + AIN-93G ^A	30.1 ± 1.1	30.9 ± 1.5	25.4 ± 2.3*	0.59	<0.001
LYC + Placebo ^B	30.7 ± 0.9	32.1 ± 1.2	26.1 ± 0.8*	0.23	<0.001
Visceral Adipose (weight as a % of body weight)					
TP + AIN-93G ^A	5.4 ± 0.6	5.0 ± 0.6	4.1 ± 0.5	0.47	0.02
LYC + Placebo ^B	5.2 ± 0.5	5.9 ± 0.6	4.0 ± 0.6*	0.19	0.002
Uterus (weight as a % of body weight)					
TP + AIN-93G ^A	0.6 ± 0.05	0.3 ± 0.05*	0.4 ± 0.05*	<0.001	0.005
LYC + Placebo ^B	0.6 ± 0.05	0.3 ± 0.05*	0.4 ± 0.05*	<0.001	<0.001
Ovaries (weight as a % of body weight)					
TP + AIN-93G ^A	0.06 ± 0.006	0.04 ± 0.006*	0.06 ± 0.007	0.008	0.50
LYC + Placebo ^B	0.06 ± 0.006	0.05 ± 0.006	0.06 ± 0.006	0.10	0.78
Liver (weight as a % of body weight)					
TP + AIN-93G ^A	3.3 ± 0.07	3.4 ± 0.08	3.8 ± 0.06	0.45	<0.001
LYC + Placebo ^B	3.4 ± 0.07	3.4 ± 0.07	3.7 ± 0.06	0.96	0.006 [#]
Kidney (weight as a % of body weight)					
TP + AIN-93G ^A	0.96 ± 0.02	0.92 ± 0.02	1.03 ± 0.01	0.22	0.002
LYC + Placebo ^B	0.97 ± 0.03	0.90 ± 0.03	1.03 ± 0.02	0.07	0.008
Spleen (weight as a % of body weight)					
TP + AIN-93G ^A	0.31 ± 0.02	0.31 ± 0.02	0.36 ± 0.01	0.57	<0.001
LYC + Placebo ^B	0.28 ± 0.02	0.31 ± 0.02	0.36 ± 0.01	0.79	<0.001

^A TP + AIN-93G= tomato powder and AIN-93G-fed animals; ^BLYC + Placebo= lycopene beadlet and Placebo beadlet-fed animals; * Indicates significantly different from WT; **P-values represent comparison between WT mice and each KO mouse genotype individually; [#] signifies a diet effect was seen in addition to genotype; n=20-22/genotype/treatment

Table 9: Body and organ weights of the 4 day feeding study.

Treatment	WT	CMO-I KO	CMO-II KO	P-value**	
				CMO-I KO	CMO-II KO
Body Weight (g ± SEM)					
TP + AIN-93G ^A	27.2 ± 0.7	29.3 ± 0.7	24.2 ± 0.8*	0.59	<0.001
LYC + Placebo ^B	28.0 ± 0.9	29.7 ± 1.2	23.3 ± 0.7*	0.23	<0.001
Visceral Adipose (weight as a % of body weight)					
TP + AIN-93G ^A	3.0 ± 0.4	4.2 ± 0.4*	2.8 ± 0.4	0.03	0.99
LYC + Placebo ^B	3.5 ± 0.3	4.0 ± 0.3	2.9 ± 0.3	0.30	0.25
Uterus (weight as a % of body weight)					
TP + AIN-93G ^A	0.76 ± 0.05	0.3 ± 0.05*	0.41 ± 0.06*	<0.001	<0.001
LYC + Placebo ^B	0.60 ± 0.04	0.4 ± 0.04*	0.52 ± 0.04	<0.001	0.18
Ovaries (weight as a % of body weight)					
TP + AIN-93G ^A	0.08 ± 0.01	0.04 ± 0.01*	0.06 ± 0.01	<0.001	0.92
LYC + Placebo ^B	0.07 ± 0.004	0.06 ± 0.007*	0.07 ± 0.004*	<0.001	0.01
Liver (weight as a % of body weight)					
TP + AIN-93G ^A	3.6 ± 0.09	3.7 ± 0.09	3.8 ± 0.06	0.37	0.18
LYC + Placebo ^B	3.6 ± 0.08	3.7 ± 0.08	3.6 ± 0.09	0.41	0.67
Kidney (weight as a % of body weight)					
TP + AIN-93G ^A	1.1 ± 0.02	1.0 ± 0.02	1.1 ± 0.01	0.002	0.59
LYC + Placebo ^B	1.1 ± 0.02	0.97 ± 0.02	1.0 ± 0.02	0.004	0.43
Spleen (weight as a % of body weight)					
TP + AIN-93G ^A	0.30 ± 0.02	0.29 ± 0.02	0.38 ± 0.02	0.49	0.003
LYC + Placebo ^B	0.30 ± 0.01	0.28 ± 0.01	0.34 ± 0.02	0.52	0.14

^A TP + AIN-93G= tomato powder and AIN-93G-fed animals; ^BLYC + Placebo= lycopene beadlet and placebo beadlet-fed animals; * Indicates significantly different from WT; **P-values represent comparison between WT mice and each KO mouse genotype individually; n=20-22/genotype/treatment

Table 10: Hepatic lipid accumulation in 30 day fed CMO-I KO mice compared to WT mice¹.

Diet	WT	CMO-I KO	P-value		
			Genotype	Diet	Interaction
AIN-93 G	65.9 ± 4.4	64.6 ± 5.2	0.43	0.68	0.40
Tomato Powder	58.2 ± 2.9	67.9 ± 5.8			
Placebo Beadlet	61.0 ± 3.6	76.1 ± 4.9	<0.05	0.17	0.52
Lycopene Beadlet	58.3 ± 3.6	68.0 ± 6.3			

¹ Weights are represented as mg lipid/g liver; n=9-11/genotype/diet

Table 11: Hepatic lipid accumulation in 30 day fed CMO-II KO mice compared to WT mice¹.

Diet	WT	CMO-II KO	P-value		
			Genotype	Diet	Interaction
AIN-93 G	65.9 ± 4.4	90.2 ± 7.7	<0.001	0.16	0.46
Tomato Powder	58.2 ± 2.9	80.3 ± 9.9			
Placebo Beadlet	61.0 ± 3.6	90.2 ± 10.5	<0.001	0.08	0.32
Lycopene Beadlet	58.3 ± 3.6	74.8 ± 7.0			

¹ Weights are represented as mg lipid/g liver; n=9-11/genotype/diet

Table 12: Hepatic lipid accumulation in 4 day fed CMO-I KO mice compared to WT mice¹.

Diet	WT	CMO-I KO	P-value		
			Genotype	Diet	Interaction
AIN-93 G	53.3 ± 3.4	62.0 ± 5.4	0.01	0.49	0.27
Tomato Powder	47.5 ± 1.5	63.8 ± 5.0			
Placebo Beadlet	48.8 ± 2.2	61.8 ± 3.5	0.01	0.61	0.24
Lycopene Beadlet	51.1 ± 2.8	56.1 ± 4.3			

¹ Weights are represented as mg lipid/g liver ± SEM; n=7/genotype/diet

Table 13: Hepatic lipid accumulation in 4 day fed CMO-II KO mice compared to WT mice¹.

Diet	WT	CMO-II KO	P-value		
			Genotype	Diet	Interaction
AIN-93 G	53.3 ± 3.4	62.2 ± 5.3	0.001	0.38	0.68
Tomato Powder	47.5 ± 1.5	67.7 ± 3.2			
Placebo Beadlet	48.8 ± 2.2	65.8 ± 3.4	0.003	0.16	0.14
Lycopene Beadlet	51.1 ± 2.8	73.4 ± 3.9			

¹ Weights are represented as mg lipid/g liver ± SEM; n=7/genotype/diet

Table 14: Hepatic total cholesterol accumulation in 4 day fed CMO-II KO mice compared to WT mice¹.

Diet	WT	CMO-II KO	P-value		
			Genotype	Diet	Interaction
AIN-93 G	81.0 ± 7.8	92.1 ± 5.5	0.92	0.96	0.33
Tomato Powder	92.6 ± 16.4	81.5 ± 4.0			
Placebo Beadlet	70.3 ± 4.9	95.5 ± 5.3	0.003	0.15	0.32
Lycopene Beadlet	74.0 ± 9.9	115.3 ± 6.1			

¹ Weights are represented as mg cholesterol/dL lipid solution ± SEM; n=7/genotype/diet

Table 15: Serum total cholesterol accumulation in 30 day fed CMO-I KO mice compared to WT mice¹.

Diet	WT	CMO-I KO	P-value		
			Genotype	Diet	Interaction
AIN-93 G	122 ± 5.4	101 ± 7.0	0.002	0.32	0.95
Tomato Powder	130 ± 4.2	106 ± 4.6			
Placebo Beadlet	126 ± 7.7	108 ± 15.4	0.14	0.07	0.92
Lycopene Beadlet	146 ± 9.4	128 ± 7.7			

¹ Weights are represented as mg cholesterol/dL serum ± SEM; n=7-8/genotype/diet

Table 16: Serum total cholesterol accumulation in 30 day fed CMO-II KO mice compared to WT mice¹.

Diet	WT	CMO-II KO	P-value		
			Genotype	Diet	Interaction
AIN-93 G	122 ± 5.4	99.7 ± 3.3	<0.001	0.93	0.25
Tomato Powder	130 ± 4.2	92.2 ± 7.5			
Placebo Beadlet	126 ± 7.7	104 ± 6.0	<0.001	0.62	0.03
Lycopene Beadlet	146 ± 9.4	95.4 ± 7.9			

¹ Weights are represented as mg cholesterol/dL serum ± SEM; n=7-8/genotype/diet

Table 17: Serum total cholesterol accumulation in 4 day fed CMO-I KO mice compared to WT mice¹.

Diet	WT	CMO-I KO	P-value		
			Genotype	Diet	Interaction
AIN-93 G	119 ± 9.7	110 ± 5.5	0.99	0.45	0.19
Tomato Powder	115 ± 7.1	124 ± 4.3			
Placebo Beadlet	123 ± 8.9	120 ± 8.3	0.01	0.29	0.03
Lycopene Beadlet	133 ± 7.7	94.1 ± 5.6			

¹ Weights are represented as mg cholesterol/dL serum ± SEM; n=7-8/genotype/diet

Table 18: Serum total cholesterol accumulation in 4 day fed CMO-II KO mice compared to WT mice¹.

Diet	WT	CMO-II KO	P-value		
			Genotype	Diet	Interaction
AIN-93 G	119 ± 9.7	90.8 ± 6.0	0.001	0.57	0.96
Tomato Powder	115 ± 7.1	86.0 ± 6.7			
Placebo Beadlet	123 ± 8.9	93.4 ± 3.9	<0.001	0.47	0.56
Lycopene Beadlet	133 ± 7.7	94.4 ± 4.8			

¹ Weights are represented as mg cholesterol/dL serum ± SEM; n=7-8/genotype/diet

Chapter III

Discussion and Future Directions

Discussion

Consumption of fruits and vegetables is an essential part of a healthy diet and researchers have suggested that the carotenoids found in these food components have health benefits that go beyond vitamin A production (2). Although many of the health benefits of carotenoids have been implied, the metabolism of carotenoids within the organs is largely unknown and is an active area of research. Our lab has long term interest in the many actions of tomato carotenoids, especially lycopene. We are one of a few labs in the world to have CMO-I KO and CMO-II KO mouse colonies that can be used as models to investigate the metabolism of lycopene *in vivo*. In the past, our lab has been primarily focused on male CMO-I KO and CMO-II KO mice and had not extensively evaluated female CMO-I KO and CMO-II KO mice. Based primarily on work initially-published in CMO-I KO mice (47), we wanted to focus on lipid metabolism in our female KO mice.

We fed 29-31 week old female CMO-I KO, CMO-II KO and WT mice carotenoid-containing diets (tomato powder or lycopene beadlet diet) or their respective controls (AIN-93G or placebo beadlet diets) for either 30 or 4 days. We analyzed the body and organ weights in the 30 day study and found CMO-II KO mice to be smaller than WT mice in body weight and reproductive organ weights, as percent body weight, but higher weights in most non-reproductive organs compared to WT mice. CMO-I KO mice were not different in body weight compared to WT mice, but as was seen in CMO-II KO mice, their reproductive organ weights were significantly smaller.

We were also interested in hepatic lipid accumulation in CMO-I KO and CMO-II KO mice. On average for both the 30 day and 4 day feeding study, CMO-I KO and CMO-II KO mice had significantly greater accumulation of hepatic lipids compared to WT mice. Contrary to what has been previously published on hepatic lipids for these genotypes (10, 47), we did not observe hepatic steatosis in our CMO-II KO mice and only saw minimal hepatic lipidosis in 25% of our CMO-I KO mice. Mice were fed a diet with low, but not clinically deficient, levels of vitamin A with normal fat levels, whereas in the other studies that reported hepatic steatosis in these strains, diets were either high in fat or deficient in vitamin A. We next wanted to investigate hepatic and serum cholesterol in our animals and found that CMO-II KO mice were significantly different from WT mice in hepatic and serum cholesterol levels in most diet groups. Finally, mRNA expression of PPARs and selected target genes were investigated. We found diet to have some impact on these genes. Tomato powder modulated the mRNA expression of PPAR γ and its target gene, as well as PPAR α .

Based on the results in this thesis we conclude that CMO-I KO and CMO-II KO mice have substantial phenotypic differences compared to WT mice in many measures of lipid status. However, feeding either a tomato powder or lycopene-supplemented diets to these animals had marginal effects compared to the effects seen with ablation of either carotenoid cleavage enzyme. It is clear that these enzymes, CMO-I and CMO-II, have functions that go beyond cleaving carotenoids and may play an important role in lipid metabolism.

Future Directions

In an assessment of the small amount of published literature, it is apparent that vitamin A levels in the rodent diet play an important part in the results seen in lipid metabolism in both the CMO-I KO mice and CMO-II KO mice (10, 24, 47). The hepatic vitamin A levels in our mice

were not measured and it would be interesting to know the differences between these genotypes regarding hepatic storage of vitamin A. Additionally, it would be of interest to measure the levels of lycopene metabolites in our tomato powder and lycopene-fed CMO-I KO and CMO-II KO mice to determine the *in vivo* metabolism of lycopene in these animals. It would also be interesting to compare the differences in lycopene metabolites between animals fed tomato powder or lycopene within the same genotype.

Based on discrepancies in the literature regarding CMO-I KO and CMO-II KO mice and hepatic steatosis, there are at least two studies that would help clarify these results. In order to determine if hepatic steatosis could be prevented, or reversed, with introduction of carotenoids into the diet, two different studies would need to be performed. For one study, CMO-I KO, CMO-II KO and WT mice (n=720; 10 mice per genotype x 3 genotypes x 4 vitamin A level x 2 fat levels x 3 treatments), at 20 weeks of age, would receive a high fat (30% w/w as used in Hessel et al.) or normal (10% w/w) fat diet with different levels of vitamin A (deficient 150, low 1500, recommended 2400, high 14,000 IU/kg diet), with carotenoids (tomato or lycopene beadlets) or without (AIN-93G diet supplemented with placebo beadlets), and after 8 weeks of feeding the animals would be sacrificed (ages and length of feeding based on Hessel et al.). Hepatic lipid analysis and plasma lipids could be compared to determine if carotenoids, in conjunction with the different vitamin A status, could alter the fatty liver phenotype seen in these mice (10, 47).

In the other study, animals (n=; 360, 10 per genotype x 3 genotypes x 4 vitamin A levels x 3 treatments) would be fed a high fat diet with low level of vitamin A (level may need to change if hepatic steatosis does not develop before placement onto experimental diets) after weaning, and after a predetermined number of weeks of feeding (determined by onset of hepatic

steatosis in these animals), mice would be placed on a control diet (each level of vitamin A, low fat AIN-93G supplemented with placebo beadlets) or a carotenoid-containing diet (each level of vitamin A, low fat; tomato powder supplemented with placebo beadlets or lycopene beadlet supplemented diet). These animals would be sacrificed after 8 weeks of feeding and liver lipids and plasma lipids would be compared to determine if lycopene or tomato powder could reverse hepatic steatosis.

These studies would help to determine if the fatty liver phenotype of CMO-I KO mice and CMO-II KO mice in other studies was a result of vitamin A status or high fat feeding. By supplementing various levels of vitamin A to a high fat diet with or without carotenoids we would be able to determine which dietary factor(s) is predominantly contributing to this phenotype. We may also be able to determine at what level of vitamin A these carotenoids are able to prevent the onset of fatty liver. Additionally, by introducing lower fat diets to animals that already have hepatic steatosis, and further supplementing these diets with carotenoids, we could determine if low fat feeding alone or with the addition of carotenoids could reverse this phenotype. These results would help determine if dietary modifications can reverse hepatic steatosis.

Chapter IV

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